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A QUANTITATIVE STUDY OF REACTIONS TO ELECTRICITY IN AMOEBA PROTEUS[1]

(Eleven figures)

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INTRODUCTION

IT IS well known that electrical changes profoundly affect various vital processes. Among the most striking of these is the effect on protoplasmic streaming. Becquerel (1837) concluded from the results of observations on *Chara* (1) that a constant current causes retardation and then cessation of streaming, (2) that the time required to produce these effects depends upon the strength of the current, (3) that after streaming is stopped by a weak current, it soon begins again and usually attains its original velocity but never a greater velocity than existed before excitation, and (4) that after streaming is resumed, a stronger current is required to cause it to stop again. Results confirming the first two of these conclusions were obtained by Jürgensen (1861) in *Vallisnaria*, Hörmann (1898) in *Nitella*, and Koketsu (1923) in *Chara*. Koketsu maintains that a constant current causes increase in the viscosity of the protoplasm, a view previously proposed by Chifflot and Gautier (1905) and Bersa and Weber (1922) to account for the effect produced by the current in certain other plant cells. Velten (1876) and Ewart (1903) hold that weak currents frequently accelerate streaming; and Hörmann

[1] This investigation was suggested by Professor S. O. Mast, to whom the author is indebted also for invaluable assistance and criticism, especially in the preparation of the manuscript.

(1898) contends that after cessation has been produced once, a weaker current suffices to produce it again.

The investigation of the effect of a galvanic current on protoplasmic streaming and on locomotion in the lower animals has yielded similar results. Kühne (1864) and Engelmann (1869) observed that in *Amoeba* a constant current causes cessation of streaming and that the time required to produce it depends upon the strength of the current. Kühne asserts in support of Becquerel that after a few induction shocks a stronger current is required to produce a reaction, but he holds in opposition to Becquerel that single weak shocks frequently accelerate the rate of streaming.

Verworn (1896) demonstrated that *Amoeba* orients definitely in a galvanic current and migrates toward the cathode. This has been abundantly confirmed; it is ascribed by Verworn (1896) to anodal contraction, by Carlgren (1900) to localized changes in water content within the organism due to endosmotic streaming, by Coehn and Barratt (1905) to cataphoresis, by Hirschfeld (1909) to reduction in surface tension on the cathodal side, by McClendon (1911) to increase in surface tension on the anodal side, and by Luce (1926) and Mast (1931) to local decrease in the elastic strength of the plasmagel on the cathodal side of the organism.

Luce (1926) made a detailed study concerning the processes involved in orientation to electricity in *Amoeba*. He obtained some very interesting results concerning the relation between the strength of the current and the directness of orientation. He says: "Orientation is slow and uncertain with weak currents and becomes more rapid as the current increases until direct reversal of flow takes place." He concludes: "Orientation is brought about by the formation of pseudopods on the cathodal side, the effect of the current being to cause tendencies toward solation on that side." Mast (1931) comes to similar conclusions on the basis of results obtained in observations on the effect of alternating current.

The evidence at hand shows clearly that there is great diversity of opinion concerning the action of electricity on the lower organisms. It shows that *Amoeba* orients and migrates toward the cathode and that the process of orientation is directly dependent upon the strength of the current, but it does not show the relation between the

strength of the current and the rate of migration or the reaction-time in the response to electricity. The present investigation treats of these problems and is primarily concerned with the quantitative relation between the stimulating agent and the subsequent response, as indicated by changes in the rate and direction of movement.

MATERIAL AND METHODS

All of the observations described in the following pages were made on specimens derived from a pure clone culture of *Amoeba proteus* Leidy. These amoebae were cultivated and studied in synthetic solutions of known salt content and hydrogen-ion concentration. Chemi-

TABLE I

SALT SOLUTION USED FOR CULTURING *Amoeba proteus*

$NaCl$	0.08 gram	
$NaHCO_3$	0.004 gram	
KCl	0.004 gram	
$CaCl_2$	0.004 gram	
$CaH_4(PO_4)_2$	0.002 gram	
Sorensen's phosphate mixture:		
Na_2HPO_4, M/15	2 cc.	= pH 6.64
KH_2PO_4, M/15	3 cc.	
Redistilled water to	1,000 cc.	

cals of the highest purity and water redistilled in pyrex glass were used in all solutions.

The culture method may be described as follows: A salt solution was prepared as indicated in Table I. About 100 cc. of this solution was poured into each of a number of finger bowls, after which one or two grains of polished rice and specimens of *Amoeba proteus* and *Chilomonas paramecium* were added to each. The rice soon became covered with a mold, on and among the strands of which numerous specimens of *Amoeba* and *Chilomonas* aggregated. New cultures were set up by transferring one of these grains with its mold and organisms to a finger bowl containing fresh solution.

In order to ascertain the effect of the constant electric current on locomotion and other processes in *Amoeba*, it is necessary to vary this factor in a known way without varying any other factors. This cannot be accurately done in the culture fluid, because in it the elec-

trolytic decomposition by the current is great and the general excitability of the organism is reduced. For this reason, all of the observations were made in another synthetic solution, designated as "test solution." Its composition is given in Table II. It differs from the culture solution as follows: (1) Sodium bicarbonate, sodium chloride, potassium chloride, and calcium acid phosphate are eliminated. (2) The phosphate buffer and the calcium chloride are more concentrated. (3) The ratio of Na, K, and Ca is 12:15:2, as compared with 38:9:1 in the culture solution. (4) The total salt content is only about one-half that of the culture fluid.

The experiments were carried out in a darkened room, observations being made with a compound microscope. The electricity used

TABLE II

SALT SOLUTION USED IN THE EXPERIMENTAL TESTS

$CaCl_2$	0.008 gram	
Sorensen's phosphate mixture:		
Na_2HPO_4, M/15	4 cc.	= pH 6.64
KH_2PO_4, M/15	6 cc.	
Redistilled water to	1,000 cc.	

was drawn from a 110-volt direct-current lighting circuit, passed through two MacLagan rheostats to a reversing switch, then through a mercury contact key to sheet-platinum electrodes. A very accurate Rawson milliammeter was placed in line in front of the reversing switch (Fig. 1, *A*). The observation slide used in these tests contained a rectangular glass trough, 70 mm. long, 10 mm. wide, and 5 mm. deep; it was constructed by cementing strips of glass slide on the surface of a glass slide. Two porcelain plates, 0.4 mm. in thickness, were cemented across the trough, dividing it into three compartments—two for the electrodes and one for the amoebae (Fig. 1, *B* and *C*). Hereafter the center compartment of this trough will be referred to as the "observation compartment" and the end ones as the "electrode compartments."

In making the experiments, the three compartments on the observation slide were filled with the test solution; then about twenty amoebae were taken from a stock culture and put into a pyrex glass

beaker containing redistilled water and left 15 minutes.[2] By the end of this time, nearly all of the amoebae were radiate in form; these were removed and transferred through four changes of test solution, allowing them to remain 15 minutes in the last for adjustment. By

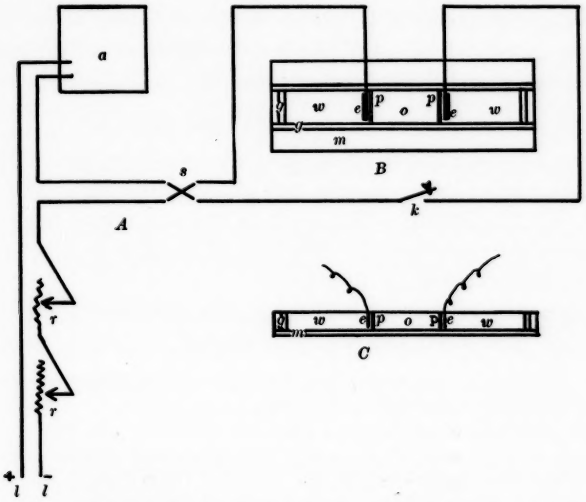

FIG. 1.—*A*, Diagram showing arrangement of apparatus used in the study of reactions to a galvanic current in *Amoeba*. *B*, The observation slide used in this study, viewed from above. *C*, The same viewed from the side. *l-l*, Current source; *r-r*, rheostats in series; *a*, ammeter; *s*, reversing switch; *k*, mercury contact key; *e-e*, electrodes; *w-w*, electrode compartments; *o*, observation compartment; *p-p*, porcelain plates; *m*, microscope slide; *g-g*, glass strips forming walls of trough.

this time many of them were fairly monopodal; two to five of these were then transferred to the middle of the center compartment on the observation slide. A cover glass was now placed over this compartment and the observation slide put upon the stage of the compound microscope, after which the electrodes were inserted into the solution in the end compartments. As soon as the amoebae became attached to the bottom, a specimen which was monopodal and moving

[2] The wash in redistilled water was used only in the experiments on the rate of locomotion. There is some indication that it may play a rôle in causing the amoebae to assume the monopodal form more quickly and to retain it for a longer period of time.

in the desired direction was selected, then the polarity of the electrodes was adjusted as desired by means of the reversing switch. The current[3] was now turned on by closing the contact key, and the reaction of the specimen observed.

Under favorable conditions the response in *Amoeba* to a constant current is sharp and precise, so that it can be clearly seen and conveniently measured. Most of the results obtained are presented in the form of figures, tables, and graphs.

LOCOMOTION IN UNSTIMULATED AMOEBAE

Before discussing the effect of electricity on movement in *Amoeba*, a brief account of locomotion in unstimulated specimens will prove helpful. For the present study, such an account should treat of the structure of the organism, the processes involved in locomotion, and the rate of locomotion.

The structure of *Amoeba proteus* and the processes involved in its locomotion have been thoroughly discussed by Mast (1926). He says (p. 411):

> An active monopodal amoeba of the proteus type may then be looked upon as consisting essentially of an elongated relatively rigid tube (plasmagel) with the one end, the anterior, covered with a thin sheet (plasmagel sheet) of porous substance having a low elastic strength, and the other, the posterior, with a thicker sheet having a higher elastic strength, a hypertonic fluid substance (plasmasol) filling the cavity of the tube, a very thin membrane (plasmalemma) of very low elastic strength covering the tube, and a thin layer of liquid between the surface membrane and the tube nearly everywhere except in the regions adjoining those where the membrane is in contact with the substratum (this layer is thick at the tip of active pseudopods and is called hyaline cap). And locomotion in such an amoeba may be looked upon as being essentially due to contraction of the thick sheet covering the posterior end, forcing the column of fluid, the plasmasol, in the tube forward against the thin sheet at the anterior end, stretching it and pushing it forward together with the surface membrane, which, owing to attachment to the substratum and to the adjoining wall of the tube, slides forward over the wall of the tube above, resulting in forward movement of the whole system.

Concerning the rate of locomotion in *Amoeba proteus*, the data are very meager. Schaeffer (1920) says that it moves about 600 μ per

[3] The strength of the current was controlled by means of the rheostats; it is expressed in delta (δ), a unit which indicates a current of 1/1,000 milliampere per square millimeter of cross-sectional area of the solution in the observation compartment.

minute. Schwitalla (1924) made a detailed study on the effect of temperature on the rate of locomotion, and states that the average rate of all the amoebae studied at various temperatures was 625 μ per minute. This must, however, be a misprint, since none of the data presented in his paper shows a rate greater than 250 μ per minute. Hopkins (1928), in observations on the effect of hydrogen-ion concentration, obtained a maximum rate of about 150 μ per minute. Pantin (1924) maintains that the maximum rate in "limax," a marine amoeba, is about 180 μ per minute and that, if the conditions of the medium are kept constant, the rate in any individual is constant to within 1–5 per cent for at least 24 hours. Schwitalla contends that this does not obtain for *Amoeba proteus*. He says that in this form the rate usually varies greatly, only rarely remaining the same in two successive minutes and frequently changing from zero to the maximum, or the converse, in a very few minutes. By referring to Figure 3 in Schwitalla's paper, it will be seen that there was great variation in the form of the amoebae used; i.e., some were nearly monopodal, others bipodal, and still others multipodal. It may be, therefore, that the rate observed by him was specifically correlated with the form. If this is true, variation in rate can be greatly reduced by selecting amoebae of the same form. If this can be done, it will obviously greatly facilitate observations on the effect of environmental factors on the rate. Further observations on the rate of locomotion in unstimulated specimens were consequently made as follows:

Two to ten amoebae, prepared as described above, were put into test solution in the observation compartment. As soon as they became attached to the bottom, a monopodal specimen was selected; its rate of locomotion was then ascertained by projecting the image of the specimen upon a sheet of paper by means of a camera lucida, marking the position of the posterior end of the organism at intervals of 1 minute over a long period of time, and measuring the distance between successive positions with a metric rule. The image as projected upon the paper was magnified 85 times, making the rate recorded 85 times the actual rate.

The rate of locomotion was thus ascertained for numerous specimens, but the records of only ten are used as the basis for this dis-

cussion. These ten amoebae were observed at minute intervals for a total of 177 minutes, so that, on an average, each amoeba was under observation 18 minutes.

The results obtained are presented in Figure 2 and Table III. Figure 2 is the actual camera lucida record of locomotion obtained in

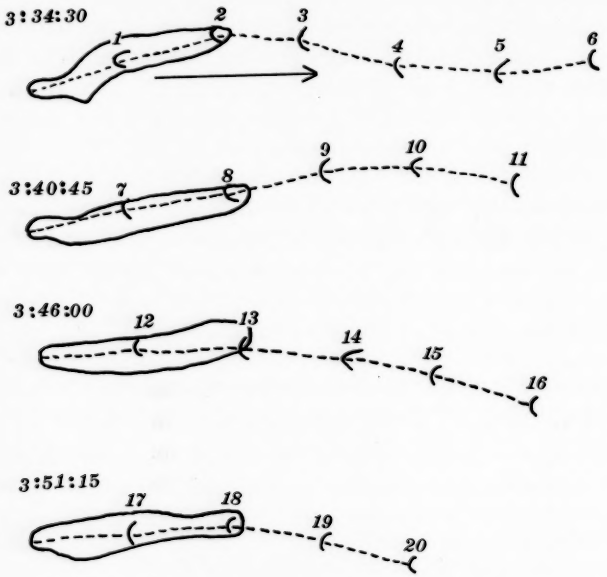

FIG. 2.—Camera lucida drawings showing record of locomotion in an unstimulated amoeba. Arrows, direction of locomotion; dotted line, path taken; curved cross-lines, positions of the posterior end at intervals of 1 minute.

one specimen. The figure shows that the record of the positions of the organism is clear, the method of measurement accurate, and the rate of locomotion uniform. In fact, in 20 consecutive minutes the mean apparent rate of locomotion in this specimen was 21.47 mm. per minute, with a standard deviation of only 1.156 mm. per minute, or 5.38 per cent.

Table III contains the average of the results obtained in ten specimens. By referring to the table, it will be seen at once that the rate of locomotion over the whole period of time was remarkably con-

stant; indeed, the mean apparent rate was 20.7 mm. per minute, with a standard deviation of only 0.74 mm. per minute, or 3.57 per cent. It may be of interest to add that the average actual rate of locomotion in these ten unstimulated monopodal amoebae was about 250 μ per minute—less than one-half that given by Schaeffer.

The results presented indicate clearly that the process of ascertaining the rate of locomotion in *Amoeba proteus* is greatly simplified by using monopodal specimens and that the rate in such specimens in constant conditions is remarkably constant.

TABLE III

AVERAGE APPARENT RATE OF LOCOMOTION IN TEN UNSTIMULATED MONOPODAL AMOEBAE AT 19°–22° C.

To obtain actual rate in millimeters per minute, divide apparent rate by 85.
Mean apparent rate, 20.7 mm. per minute.
Standard deviation, 0.74 mm. per minute, or 3.57 per cent.

Periods (1 Minute Each)	Rate (Millimeters per Minute)	Periods (1 Minute Each)	Rate (Millimeters per Minute)
1	21.2	13	20.9
2	21.3	14	21.3
3	20.4	15	19.8
4	21.4	16	21.1
5	21.1	17	19.8
6	20.8	18	20.2
7	22.2	19	19.5
8	21.3	20	20.0
9	21.5	21	20.6
10	20.8	22	20.0
11	20.6	23	19.5
12	20.2		

EFFECT OF CONSTANT CURRENT DENSITY ON THE RATE OF LOCOMOTION

Having now demonstrated that under certain conditions the rate of locomotion in *Amoeba proteus* is fairly uniform over a considerable period of time, we are in a better position to consider the effect of a galvanic current on it. Let us consider first the effect produced by currents of constant density and then that produced by sudden changes in current density. Observations on the rate of locomotion in currents of constant density were made as follows:

Two to ten amoebae prepared as described above were transferred to the middle of the observation compartment and observed until

one was found which was moving in monopodal form toward the cathode. The rate of movement at the posterior end of this specimen was now recorded, by the camera lucida method, each minute for 3 or 4 minutes; the circuit was then closed, after which the rate was recorded each minute as long as the specimen continued to move in

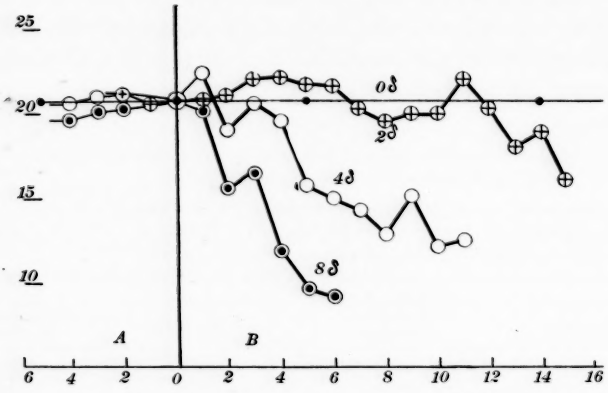

FIG. 3.—Graphs showing comparative effect of different densities of current on the rate of locomotion. Abscissae, time in minutes, (*A*) before and (*B*) during the passage of current; ordinates, apparent rate of locomotion. To obtain actual rate in millimeters per minute, divide apparent rate by 85.

monopodal form toward the cathode.[4] The distances traveled per minute before and during the passage of the current were then measured and compared. In this manner the effect of the current on the rate of locomotion was ascertained in numerous specimens.

The results obtained are presented in Figure 3. In this figure each curve represents the average apparent rate obtained in ten specimens in the stated current density. The curves are superimposed, so that at the time when the circuit was closed they coincide on the

[4] In the experiments requiring prolonged passage of current, the porcelain plates did not entirely prevent the products of electrolytic decomposition about the electrodes from diffusing into the observation compartment. These products increase the hydrogen-ion concentration of the solution surrounding the amoebae and thereby greatly affect their activities. Diffusion of these products into the observation compartment was greatly delayed by placing a fresh agar strip, 2–5 mm. thick, in each electrode compartment between the electrode and the porcelain plate before each test.

straight line; this line represents the average apparent rate calculated from all the readings obtained in ten unstimulated individuals.

The figure shows that the rate of locomotion in all cases before the circuit was closed was fairly uniform; that in a weak current (2δ), it increased above the average and remained so for 6 minutes, then gradually declined; that in a medium current (4δ), it increased above the average during the first minute, then declined gradually, but more rapidly than in the weak current; and that in a strong current (8δ), it declined slightly during the first minute, then rapidly in succeeding minutes. This clearly indicates that both the strength and the duration of the current are effective in producing changes in the rate of locomotion.

The most conspicuous feature of these curves is the ultimate trend downward; less conspicuous, but equally significant, is the initial trend upward. The question now arises as to how the current acts on the organism to produce these opposite effects on the rate of locomotion.

Numerous investigators have observed that, during the passage of a weak constant current, *Amoeba* first contracts and becomes globular, then expands and resumes its original form, and sometime later undergoes another contraction, which continues until the organism disintegrates. These changes in form greatly influence the rate of movement of different parts of the amoeba. In the contraction of a monopodal amoeba, it is evident that the rate of movement of the posterior end will increase rapidly at first, then gradually fall to zero as the globular form is approximated, and that in subsequent expansion the rate of the posterior end will gradually increase as the monopodal form is approximated, at which time the original rate will be restored. It is evident, also, with such a system that a uniform rate of movement over a period of time is possible only when the organism is in a monopodal form, since locomotion does not occur in globular amoebae.

On the basis of these facts, a sharp rise in a curve, followed by a sharp decline, indicates an injurious contraction of the organism; a gradual rise, followed by a plateau, indicates an increase in the rate of locomotion of the organism as a whole; and a gradual decline, followed by a plateau, indicates either a decrease in rate or an elonga-

tion of the individual. All of these principles are illustrated in the 2δ curve, where the initial rise and plateau show an increased rate, the gradual fall and plateau a decreased rate, and the second rise and fall contraction leading to disintegration. Further evidence in support of this interpretion will be presented in the following section.

As stated in the introduction, Becquerel (1837) holds that in *Chara* the passage of a galvanic current never causes increase in the rate of protoplasmic streaming. Contrary results were obtained by Velten (1876) and Ewart (1903) in plants and by Kühne (1864) in sluggish amoebae. The results presented above are also to the contrary and offer quantitative evidence that in *Amoeba* a weak constant current does increase the rate of protoplasmic streaming, that is, locomotion. Discussion concerning the processes involved in the increase in the velocity of movement in *Amoeba* will be deferred until certain other data have been presented.

The results as a whole show clearly that a weak constant current causes an initial increase in the rate of locomotion in *Amoeba proteus*, that this increase is later followed by a decline, and that the stronger the current the shorter the time required to cause the increase and the subsequent decrease in the rate of locomotion.

EFFECT OF SUDDEN CHANGE IN CURRENT DENSITY ON THE RATE OF LOCOMOTION

It was repeatedly observed in the tests just reported that the rate of movement of the posterior end in *Amoeba* suddenly increases immediately after the circuit is closed and that this effect becomes more evident as the current density increases. A detailed study was made therefore to ascertain more precisely at what time after making the current this increase in rate occurs and the relation between the magnitude of the increase and the strength of the current.

The methods used in ascertaining these relations are essentially like those used in the experiments described above. The amoebae and the observation slide were similarly prepared, after which the rate of locomotion was ascertained by means of drawings made with a camera lucida. In these tests, however, the positions of both anterior and posterior ends of the organisms were recorded, and the records were made at quarter-minute intervals. Not only were the posi-

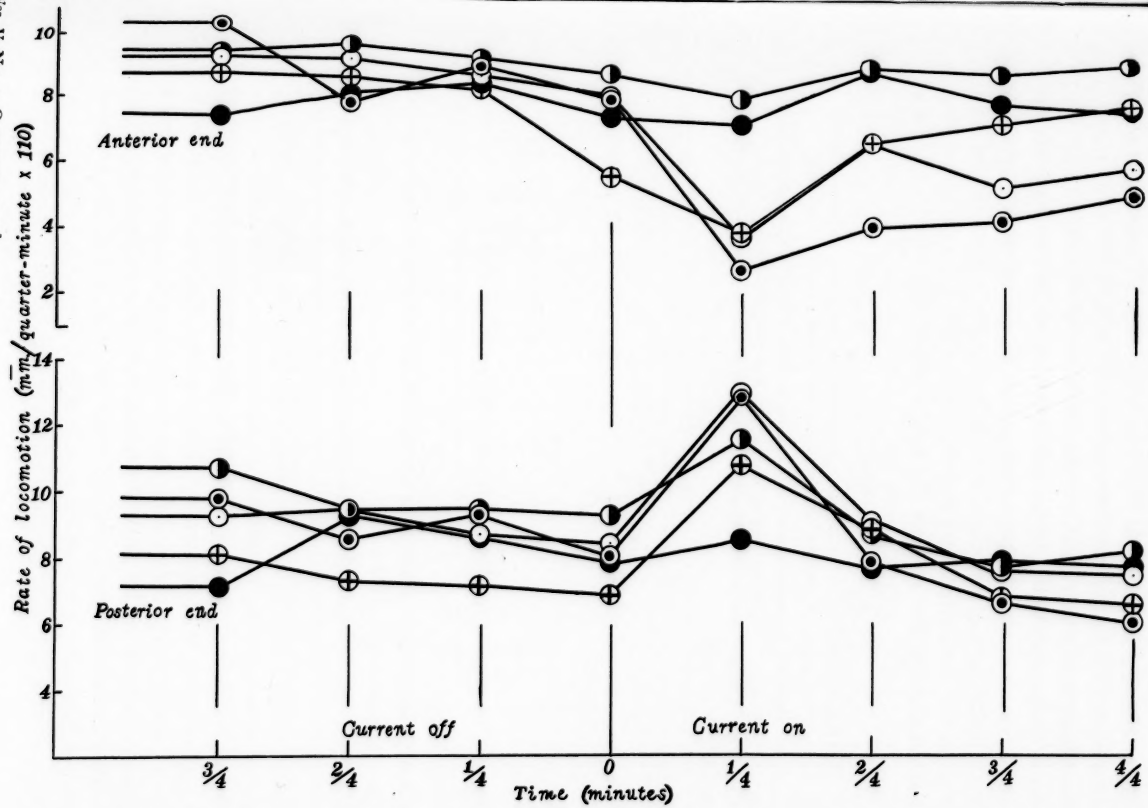

FIG. 4.—Graphs showing the immediate effect of different densities of current on the rate of movement at the two ends of amoebae. Upper group, anterior end; lower group, posterior end; ●, current 1δ; ◑, 4δ; ⊕, 8δ; ⊙, 12δ; ◉, 16δ; abscissae, time in minutes; ordinates, apparent rate of movement in millimeters per quarter-minute. To obtain actual rate in millimeters per minute, divide apparent rate by 110 and multiply by 4.

tions recorded more frequently, but they were also recorded more accurately; the latter was accomplished by means of a more powerful lens system, which gave a magnification of 110 diameters. Because of the narrowness of the field of vision with this magnification and because of the disconcerting action of strong currents on the monopodal form of amoebae, each individual was observed for only 2 minutes—1 minute before making the current and 1 minute during the passage of the current. In this manner the effect of sudden

TABLE IV

EFFECT OF CURRENTS OF DIFFERENT DENSITIES ON THE RATE OF LOCOMOTION AT THE ENDS OF *Amoeba* IMMEDIATELY AFTER THE CIRCUIT IS CLOSED

A, anterior end; P, posterior end; −, decrease in rate; +, increase in rate; numbers, average apparent distance traveled in millimeters per quarter-minute.

To obtain actual rate in millimeters per minute, divide apparent rate by 110 and multiply by 4.

Rate during Last Interval before Closing Circuit		Current Density Made	Rate during First Interval after Closing Circuit		Change in Rate during the Interval	
A	P	δ	A	P	A	P
7.25	7.85	1	7.00	8.50	−0.25	+0.65
8.65	9.25	4	7.80	11.30	−0.85	+2.05
5.44	6.85	8	3.72	10.75	−1.72	+3.90
7.95	8.40	12	3.60	12.85	−4.35	+4.45
7.85	8.00	16	2.60	12.80	−5.25	+4.80

changes in current density on the rate of movement was ascertained for numerous specimens.

The results obtained are presented in Figures 4, 5, and 6 and Table IV. In Figure 4 the rate of movement at the anterior and the posterior end of the same organisms is given in separate series of graphs, each graph in each series representing the average apparent rate obtained in ten specimens in the stated current density.

By referring to the figure, it will be seen that the rate of locomotion in all cases before the circuit was closed was fairly constant; that immediately after the current was made, it changed suddenly (i.e., within 15 seconds), as indicated by decrease in rate at the anterior end and increase in rate at the posterior end; that the changes in rate were greater the stronger the current used; and that in succeeding

periods of time the rate at either end of the organisms tended to return to that which existed before stimulation.

Some of these facts are more evident in the results presented in Figure 5, especially the uniform rate before stimulation, the sudden

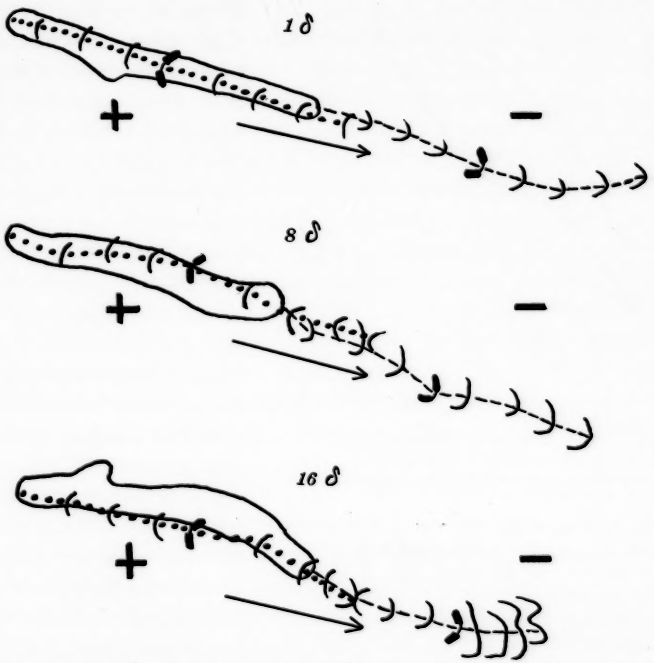

FIG. 5.—Camera lucida drawings showing the rate of locomotion in amoebae, for 1 minute before and 1 minute during the passage of current of different densities. Arrows, direction of movement; dotted line, path taken; curved cross-lines, position of the ends at successive quarter-minute intervals; heavy curved lines, position of the ends when the current was made; +, anode; −, cathode. Note that increase in rate at the anodal end and decrease in rate and expansion at the cathodal end occurred during the first quarter-minute after the current was made.

change in rate at the time of stimulation, and the tendency to resume the original rate during the passage of the current. By referring to this figure, it will be noted that the duration of the changes in rate is limited largely to the first quarter-minute after the circuit was closed and that during this period the form of the anterior end

changes considerably, i.e., it becomes broader. The significance of this will be shown presently.

The relation between the magnitude of the change in rate and the current density is still more clearly shown in Table IV, in which the distance traveled by the amoebae in the last quarter-minute before passage of current is compared with that traveled in the first quarter-minute during the passage of current. The table shows that in every density tested the rate at the anterior end decreased, while that at the posterior end increased, immediately after the current was made; that the changes in rate were greater, the stronger the current made; and that in all densities except the strongest, the change in rate was greater at the posterior than at the anterior end. These results confirm the earlier observation that on stimulation by a constant current, *Amoeba* contracts. They show, in addition, that the degree of contraction depends directly upon the current density and that most of the contractile processes involved occur at the posterior part of the organism, since the anterior end continues to move forward, although at a reduced rate.

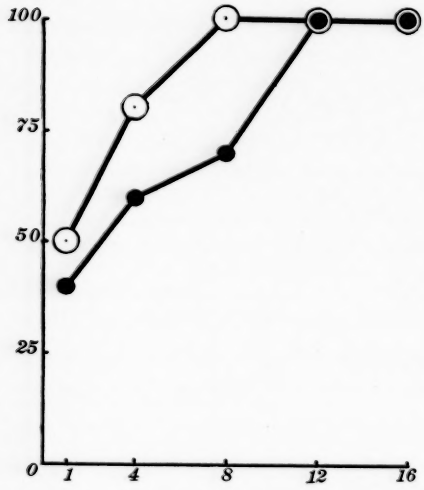

FIG. 6.—Graphs showing the relation between current density and the percentage of amoebae in which the rate of movement increased at the anodal end and decreased at the cathodal end during the first quarter-minute of passage of current. Abscissae, current density (δ); ordinates, percentage of specimens that respond; ⊙, anodal response; ●, cathodal response.

In order to bring out clearly the relation between the frequency with which these changes in rate occur in the same lot of amoebae, the percentage of specimens showing these phenomena was calculated from the individual records used in preparing Table IV. The

percentages are plotted against the corresponding current densities in Figure 6. By referring to this figure, it will be seen that in a current of 1δ, only about one-half of the specimens responded, i.e., 50 per cent showed anodal increase and 40 per cent cathodal decrease in rate; that, as the current density increased, the percentage of specimens responding increased; and that all the specimens showed anodal increase in a lower-current density than cathodal decrease. These results are similar to those obtained by Lund and Logan (1925) on coalescence of the cytoplasm of *Noctiluca* in various densities of current. The results presented in Figure 6 demonstrate, as has been so often done in the work of others, that to provoke a response, the current must exceed a certain minimal density or threshold; that the threshold varies for different individuals and for the two ends of the same individual; that it is less for anodal than for cathodal response; that it is attained for a greater percentage of specimens as the current density increases; and that it is ultimately attained for both ends of all specimens.

The foregoing results show clearly that, when subjected to a constant current, the ends of *Amoeba* undergo changes in form and in rate of movement; but they do not show what processes are involved in these phenomena. In order to ascertain more precisely the processes involved in these changes, numerous observations were made under high magnification with a Bausch and Lomb binocular compound microscope equipped with a 4 mm. objective and 15× compensating oculars. These observations were made as follows:

The amoebae used were washed as described above, and then mounted on a glass slide under a cover glass which was supported on ridges of vaseline. The cover glass was now pressed down until the drop of solution containing the amoebae filled the space beneath it; then the slide was put upon the stage of the microscope, after which the electrodes were brought near the ends of the cover glass. Contact was made between the electrodes and the solution with strips of moist filter paper. An active specimen was now selected, brought into focus, and movement carefully observed; then the current was made and the effect on the movement observed, after which the current was again broken and again made. This was repeated until the desired number of results was obtained.

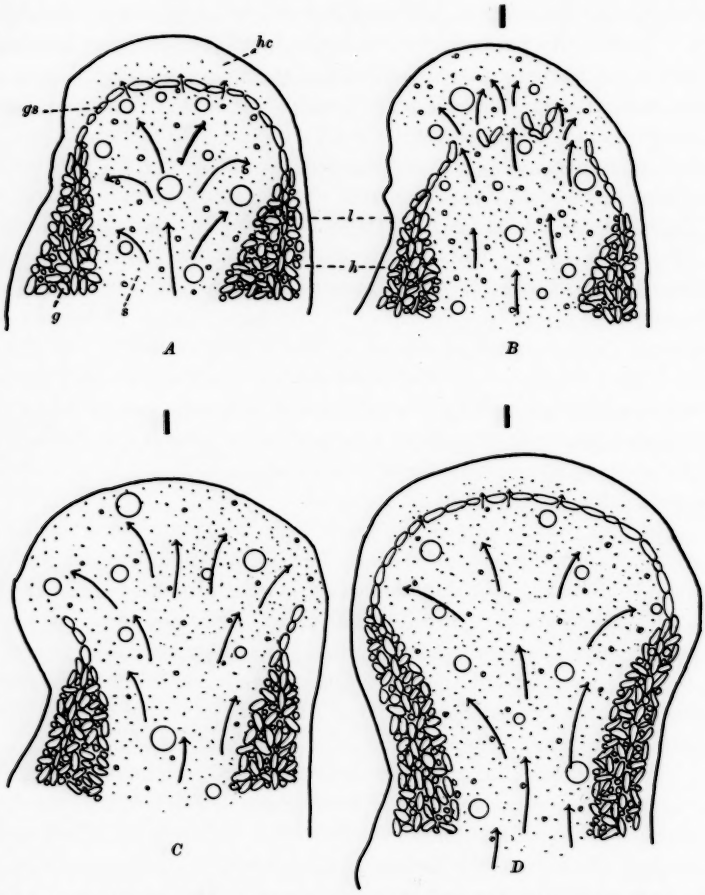

FIG. 7.—Sketches showing the changes produced by a galvanic current in the tip of a pseudopod of an amoeba which is advancing toward the cathode. *g*, Plasmagel; *s*, plasmasol; *l*, plasmalemma; *gs*, plasmagel sheet; *h*, hyaline layer; *hc*, hyaline cap; arrows, direction of flow of plasmasol; –, cathode. *A*, Anterior end showing the general relation of parts in normal locomotion (after Mast, 1926); *B*, showing break in the plasmagel sheet soon after the current is made with plasmasol streaming into the hyaline cap; *C* and *D*, later stages. Note that the plasmagel sheet disintegrates or breaks in more than one place. Note also that a new plasmagel sheet is not formed immediately, that the plasmagel tube is not extended as it is ordinarily, and that the plasmasol consequently spreads out laterally.

The results obtained are presented graphically in Figures 7 and 8. The sketches in Figure 7 represent different stages in the effect which the current produces at the anterior end of specimens moving toward the cathode. Before the current is turned on the pseudopod in monopodal specimens usually extends for considerable periods of time at a fairly uniform rate without any abrupt changes or spurts. During this time the plasmagel sheet gradually advances and remains unbroken (Fig. 7, *A*). Sometimes, however, it becomes stretched and suddenly breaks at some point, allowing the plasmasol to stream through into the hyaline cap. Then the plasmasol in contact with the plasmalemma gelates, forming a new plasmagel sheet, after which movement proceeds uniformly as before (Mast, 1926). When the current is made, the plasmagel sheet suddenly breaks, usually at several points. Since an amoeba is a turgid system, this local destruction of the plasmagel causes it to contract elsewhere, forcing the plasmasol into the hyaline cap (Fig. 7, *B*), where it spreads out laterally and, being fairly fluid, tends to assume a globular form, owing to the elasticity of the plasmalemma and the action of surface tension (Fig. 7, *C*). If the current is weak, gelation soon occurs, restoring the plasmagel sheet and the turgidity of the organism (Fig. 7, *D*). Now, however, instead of remaining intact for some time, as in unstimulated specimens, the plasmagel sheet breaks again almost immediately, after which it alternately forms and breaks at short intervals. If the current is strong or passes for some time, gelation is inhibited, preventing the formation of a new plasmagel sheet and the extension of the plasmagel tube forward. Meanwhile, the flow of plasmasol into the hyaline cap continues, with the result that, in a short time, the organism loses its monopodal form, becomes liquid and globular, and finally begins to disintegrate on the side facing the anode.

The sketches in Figure 8 represent different stages in the effect which the current produces at the posterior end of specimens moving toward the cathode. In unstimulated specimens, the plasmagel at the inner surface at the posterior end continuously solates and is forced forward by contraction of the plasmagel in that region (Fig. 8, *A*). Soon it arrives at the anterior end, where it again gelates either

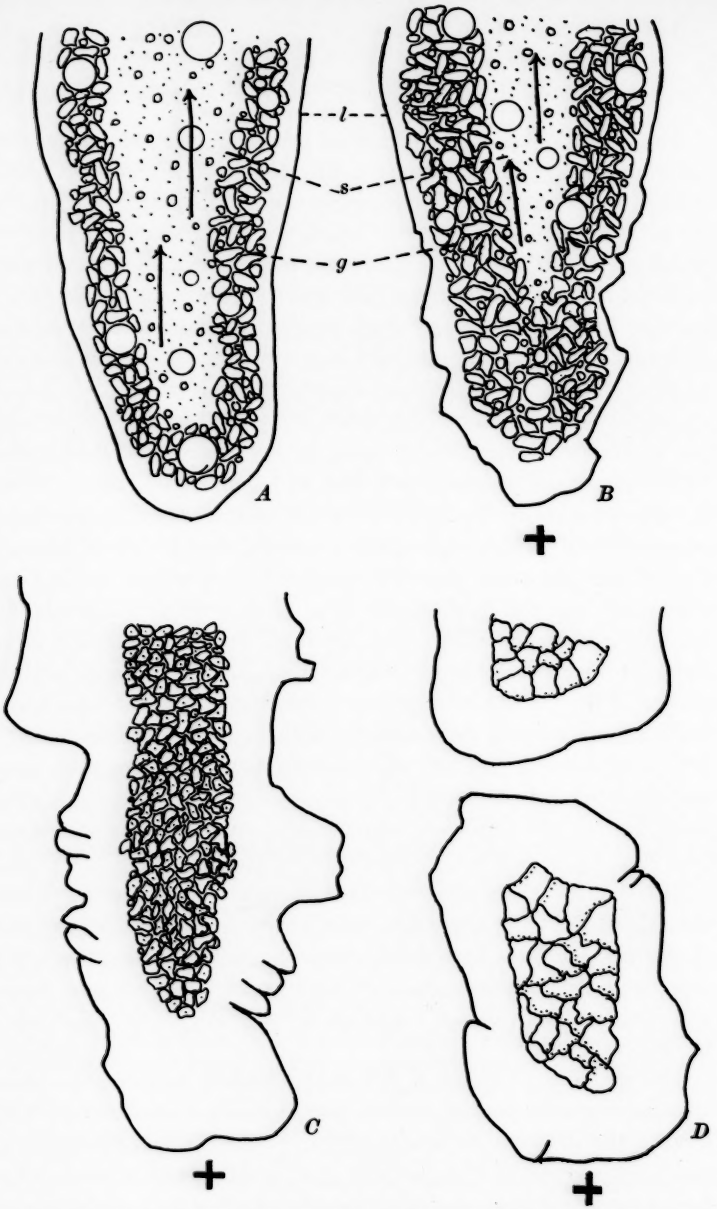

FIG. 8.—Sketches showing the changes produced by a galvanic current in the posterior end of an amoeba which is moving toward the cathode. Labels same as in Figure 7. *A*, Posterior end showing general relation of parts in normal locomotion; *B*, contraction of this end soon after the current is made, resulting in thickened plasmagel and in wrinkled plasmalemma; *C*, aggregation of the granules of the plasmagel into a dark compact mass, leaving the plasmalemma as a flaccid sac containing a hyaline liquid; *D*, pinched-off portion of the anodal end, showing the netlike structure of the plasmagel (*C* and *D* after Luce, 1927).

at the plasmagel sheet or at the forward edge of the plasmagel tube. When the current is made, the plasmagel at the posterior (anodal) end suddenly and rapidly contracts, an indirect effect following the rupture of the plasmagel sheet at the other end. If the current is weak, the period of rapid contraction continues while the plasmagel sheet is broken and ends when it is restored. If the current is strong or passes for some time, contraction of the plasmagel at the anodal end continues until the entire organism is drawn up into a dark mass, which ultimately disintegrates (Luce, 1927).

The results presented above indicate that the constant current alters the rate of locomotion in *Amoeba proteus* by definite polar actions at the ends of the organism; that these polar actions are strikingly different at the two ends; that decrease in rate of movement at the cathodal end is due to the destruction of the plasmagel sheet, allowing the pseudopod to expand laterally as well as to extend forward; that increase in rate at the anodal end is due to contraction or shrinkage of the plasmagel; that increase in the rate of locomotion of the organism as a whole is the result of increase in the frequency of breaks in the plasmagel sheet; and that the magnitude of these processes varies directly with the strength of the current.

THE REACTION-TIME IN THE RESPONSE TO ELECTRICITY

The experimental results presented in the preceding pages concern the effect of the constant current on the rate of locomotion in amoebae moving toward the cathode. In these experiments, however, owing to the fact that response to electricity occurs within a few seconds after the current is made, it was impossible to ascertain the reaction-time. It was accidentally discovered that this can be done in amoebae which are moving toward the anode.

The methods used in ascertaining the reaction-time were essentially like those used in the experiments described above. The amoebae were removed from a stock culture, transferred through four changes of test solution, and allowed to remain 15 minutes in the last for adjustment. Then two to five specimens were selected and transferred to test solution in the middle compartment on the observation slide. This compartment was now covered with a cover glass. As soon as the amoebae became attached, a specimen which was mono-

podal and moving toward the anode was selected; the circuit was then closed and the response observed.

In amoebae, which are monopodal and unstimulated, the plasmasol throughout the whole length of the organism flows toward the anterior end. If the current is made when they are moving toward the anode, the flow of the plasmasol changes; in a weak current it ceases and then reverses in direction at the cathodal end, with only a temporary cessation at the anodal end. The reversed flow continues only a few moments, after which it again reverses; streaming now proceeds in the original direction; later the organism gradually turns and becomes directed toward the cathode or rounds up and ceases to

TABLE V

REACTION-TIME IN A GIVEN SPECIMEN IN SUCCESSIVE RESPONSES TO A CURRENT OF 6δ AT INTERVALS OF 1 MINUTE

Mean reaction-time, 1.145 seconds.
Standard deviation, 0.09 second, or 7.91 per cent.

Designation of Observation	Reaction-Time (Seconds)	Designation of Observation	Reaction-Time (Seconds)
1	1.1	6	1.2
2	1.0	7	1.1
3	1.2	8	1.2
4	1.15	9	1.2
5	1.0	10	1.3

move. In a strong current, the entire plasmasol stops flowing and then reverses in direction, resulting in complete reversal in direction of movement of the organism; i.e., it now moves toward the cathode. In either case, if the circuit is opened immediately after the reverse flow at the cathodal end begins, locomotion, when it is resumed, is in the original direction; i.e., toward the anode.

These changes in the flow of the plasmasol do not take place immediately after the circuit is closed; the period which intervenes between the application of the stimulus and the response is known as the "reaction-time." In the observations reported below, a monopodal amoeba moving toward the anode was stimulated with a current of known density and the reaction-time ascertained with a stop watch. Immediately after response, the circuit was opened; then 1 minute after the preceding stimulation it was closed again; that is,

the reaction-time plus the rest period equaled 1 minute. This was repeated until the desired number of results was obtained. The reaction-time at the two ends of *Amoeba* was investigated separately; that at the posterior (cathodal) end will be considered first.

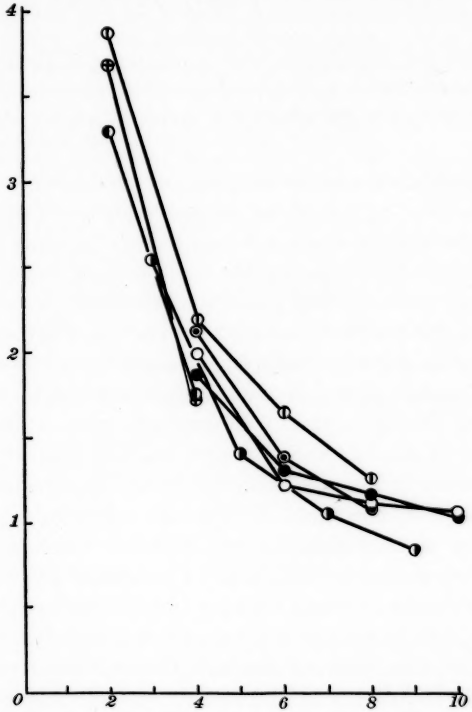

FIG. 9.—Graphs showing variation in the reaction-time in amoebae in currents of the same and of different densities. Abscissae, current density (δ); ordinates, reaction-time in seconds. Each curve is the record of one individual, and each point thereon is the average of three or more readings.

A. REACTION-TIME AT THE POSTERIOR (CATHODAL) END

1. *Individual variation in the reaction-time.*—The results obtained in a typical series of observations on the reaction-time at the posterior end of *Amoeba* are presented in Table V and Figure 9. By referring to this table, it will be seen that in ten consecutive trials the

mean reaction-time equaled 1.145 seconds, with a standard deviation of only 0.09 second, or 7.91 per cent. By referring to Figure 9, it will be seen that the reaction-time varied in different individuals in the same density; e.g., in a current of 6δ, it ranged from 1.23 seconds to 1.65 seconds, with an equally great range at several other densities.

The results presented show that in currents of the same density there is considerable variation in the reaction-time in different individuals but surprisingly little in a given individual in successive trials.

2. *Relation between reaction-time and current density.*—Having now demonstrated that by measuring certain responses in amoebae which are moving toward the anode it is possible to obtain fairly consistent quantitative results concerning the reaction-time in the response to electricity, we are in a better position to ascertain precisely the relation between the reaction-time and the current density.

Before proceeding with this study, however, it seems necessary to digress long enough to describe the procedure followed in making the observations. Owing to the facts that individual variation in the reaction-time is marked and that only a small number of readings can be obtained on a single specimen before its direction of movement or its monopodal form changes, the following procedure was adopted: The reaction-time for an individual was first ascertained in each of three successive minutes in a current of known density in order to establish the average reaction-time for the individual in that density and then in each of the next three successive minutes in a stronger current in order to establish the change in reaction-time produced by the increase in the current. In this way the reaction-time for numerous specimens was ascertained in currents of various densities.

The results obtained are presented in Tables VI and VII and Figure 10, *A*. Table VI contains the results of all the tests in considerable detail. By referring to this table, it will be seen that the average reaction-time decreased as the current density increased; e.g., in 2δ, it was 3.585 seconds; in 6δ, 1.219 seconds; and in 10δ, 0.858 second. It will also be seen that it was greater in low than in high temperatures; e.g., in 4δ, it was 1.837 seconds at 19° and 1.758 sec-

onds at 25°; and in 6δ, it was 1.280 seconds at 19° and 1.225 seconds at 25°. It will be seen, moreover, that the reaction-time was greater in amoebae which had been previously stimulated by a current than

TABLE VI

RELATION BETWEEN CURRENT DENSITY AND REACTION-TIME AT THE POSTERIOR END OF *Amoeba*

Each number represents the average for ten specimens; each pair of numbers joined by a vertical line, reaction-time on the same group of amoebae in currents of different densities; groups above slanting line, series of experiments performed at 18°–21.5° C.; groups below slanting line, those performed at 22°–27.5° C.

Current Density	Reaction-Time (Seconds)	Total Number of Readings	Average Reaction-Time (Seconds)
2	3.585	13	3.585
3	2.496	37	2.496
4	1.758 1.850 1.837	78	1.809
5	1.327 1.431	73	1.388
6	1.225 1.152 1.280	90	1.219
7	1.087 1.090	60	1.088
8	1.015 1.005	60	1.010
9	0.892	30	0.892
10	0.858	30	0.858

TABLE VII

RELATIONS BETWEEN CURRENT DENSITY AND REACTION-TIME AT THE POSTERIOR (CATHODAL) END OF *Amoeba*

(1) Current Density (i) (Microamperes)	(2) Average Reaction-Time (t) (Seconds)	(3) Quantity of Current (it)	(4) Calculated Value of K Where $K=i\sqrt{t}$	(5) Calculated Value of K Where $K=i\sqrt{t}-(0.684i)$
2	3.585	7.170	3.786	2.418
3	2.496	7.488	4.737	2.685
4	1.809	7.236	5.380	2.644
5	1.388	6.940	5.890	2.470
6	1.219	7.314	6.624	2.520
7	1.088	7.616	7.301	2.513
8	1.010	8.080	8.040	2.568
9	0.892	8.028	8.496	2.340
10	0.858	8.580	9.260	2.420

in those which had not been so stimulated; e.g., in 4δ, it was 1.850 seconds with previous stimulation and 1.837 seconds without; and in 6δ, it was 1.225 seconds with, and 1.152 seconds without previous stimulation. It will furthermore be seen that these relations do not seem to hold in currents stronger than 6δ. It should, however, be mentioned that the response in *Amoeba* to currents weaker than 3δ is irregular and indefinite and that only a small percentage of the individuals respond at all, while in currents stronger than 6δ the reaction is too rapid to be measured accurately with a stop watch.

The results presented in this table seem to indicate that decrease in temperature and previous stimulation by electricity produce the same or similar changes in *Amoeba*, since both cause increase in reaction-time. This may be due to change in viscosity or to reduced excitability or to some other change. The effects of temperature and previous stimulation on the reaction-time merit more intensive investigation.

Table VII (cols. 1 and 2) contains the average of the results presented in detail in Table VI; they are plotted as a solid curve in Figure 10, *A*, with the ordinates as reaction-time and the abscissae as current density. By referring to the table and the figure, it will be seen that the reaction-time at the cathodal end of *Amoeba* decreases as the current increases and that the curve giving the relation between reaction-time and current density closely simulates the hyperbola of equation, $xy=7$, presented in the figure by the broken curve.

The question now arises as to the quantity of electricity necessary to produce a response and the relation between this and the current density. The quantity of electricity necessary to produce a response is equal to the density of the current times the time the current must act to produce the response. The reaction-time measured above, however, is composed of two parts: the stimulation period, a time during which flow of current is necessary, and the latent period, a time during which flow of current is not necessary in order to produce a response. The reaction-time consequently does not represent the time necessary to produce a response; however, it probably closely approximates it. The product of this and the density (*it*) was consequently calculated. The results are given in Table VII, column 3.

By referring to this column, it will be seen that the product (*it*) was practically the same in all except the stronger currents tested and that the increase in these currents was probably due to the inac-

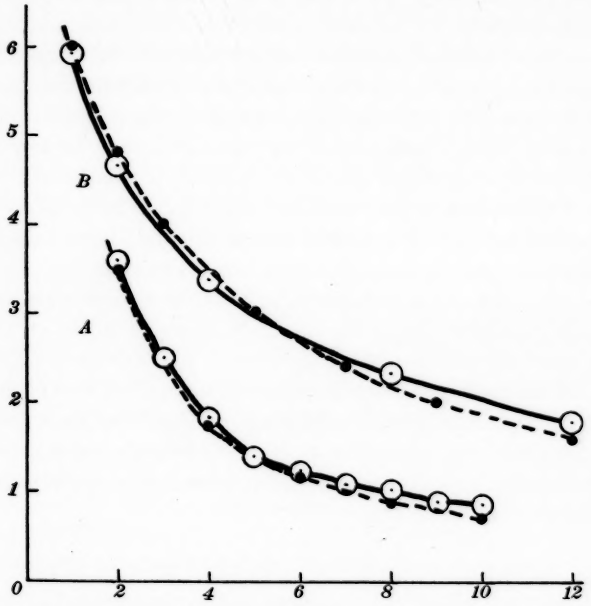

FIG. 10.—Curves showing the relation between reaction-time at the ends of amoebae and current density. Abscissae, current density (δ); ordinates, reaction-time in seconds. Solid curves, reaction-time; *A*, at posterior (cathodal) end, and *B*, at anterior (anodal) end; broken curves, hyperbolae; points on curves, points experimentally established. Note that the reaction-time decreases with increase in current density forming curves which closely simulate hyperbolae, and that the reaction-time for the anterior end is always longer than that for the posterior end in the same current.

curacy in the manipulation of the stop watch. We hope, in the near future, to ascertain the latent period over this range of densities, so as to obtain more accurately the relations in question.

It is of interest to see whether or not the response to the constant current in *Amoeba* is in accord with the law of electrical excitation which Nernst formulated in reference to response to electricity in striated muscle. His law states that for equal stimulating effect the

product of the intensity of the current (i) and the square root of its duration (t) is constant, i.e., ($i\sqrt{t}=K$). The value of K in this equation was calculated from the data presented above; it is given in Table VII, column 4. By referring to this column, it will be seen that the calculated value of K is not constant and that it increases as the current density increases. An attempt was then made to modify Nernst's equation so that the value of K would become constant. The results of this attempt are presented in the last column of the same table. This column shows that the value of K becomes nearly constant if the equation, $K=i\sqrt{t}-(0.684i)$, is used in the calculations. The number, 0.684, is the average interval between successive values of K (col. 4). The significance of this modifying factor is not known.

The tendency of the value of K to increase as the current density increases is evident also in the results of Kamada (1928) on the electro-destruction of the surface membrane of *Paramecium* and to a lesser extent in the results of Lund and Logan (1925) on coalescence of the protoplasmic films in *Noctiluca*. These cases may not, however, constitute real exceptions to Nernst's formula, since it is known that the formula only fits the results obtained in observations on muscles over a limited range of densities.

B. REACTION-TIME AT THE ANTERIOR (ANODAL) END

It was stated above that response at the anterior end in amoebae which are moving toward the anode consists in a temporary cessation of flow of plasmasol in weak currents and in cessation and then reversal in direction of flow in strong currents. This cessation does not take place immediately after the circuit is closed; i.e., there is a reaction-time.

The reaction-time at the anterior end was ascertained as in the preceding study. However, owing to the greater tendency of the anterior than of the posterior end to lose its monopodal form after stimulation, only one reading was obtained for each individual used. The reaction-time was ascertained for ten specimens in each density tested, and the average reaction-time in each calculated.

The results obtained are presented in Table VIII; they are plotted in Figure 10, *B*. By referring to the table and the figure, it will be

seen that the reaction-time decreases as the current density increases and that the curve giving the relation between reaction-time and current density closely simulates the hyperbola of equation, $xy+3y=24$, presented in the figure as a broken curve. It will also be seen that the value of K in Nernst's equation is not constant and that it increases as the current density increases. It will be seen, moreover, that if Nernst's equation is modified as described above, the value of K becomes more constant but not as constant as it does for the reaction-time at the posterior end. Greater irregularity in reaction-time would be expected at the anodal than at the cathodal end, since the response there is much less definite and precise.

TABLE VIII

RELATIONS BETWEEN CURRENT DENSITY AND REACTION-TIME AT THE ANTERIOR (ANODAL) END OF *Amoeba* AT 19.5°–22.0° C.

Current Density (i) (Microamperes)	Average Reaction-Time (t) (Seconds)	Quantity of Current (it)	Calculated Value of K Where $K=i\sqrt{t}$	Calculated Value of K Where $K=i\sqrt{t}-(0.824i)$
1	5.937	5.937	2.436	1.612
2	4.664	9.328	4.320	2.672
4	3.381	13.524	7.356	4.060
8	2.325	18.600	12.192	5.600
12	1.780	21.360	16.008	6.120
20	0.820	16.400	18.100	1.620

By comparing Table VII with Table VIII, or by comparing the two curves in Figure 10, it will be seen that the reaction-time at the posterior end was about 1 second less than that at the anterior end in each current density tested. This indicates clearly that the current produces its effect more quickly or more efficiently at the posterior (cathodal) than at the anterior (anodal) end of *Amoeba*, although the plasmagel is considerably thicker in the former than in the latter.

C. THE NATURE OF THE OBSERVED RESPONSE

The foregoing results support the view that the constant current causes solation of the plasmagel at the cathodal surface, but they do not show what occurs at the anodal surface. Moreover, they do not show the relation between the responses at the two ends of the organism. Accordingly, numerous observations were made to ascertain the nature and the relation of these responses.

The salient features in the results of these observations are presented in Figure 11, in which the sketches represent successive stages in the response to a constant current in *Amœba*, as indicated by the

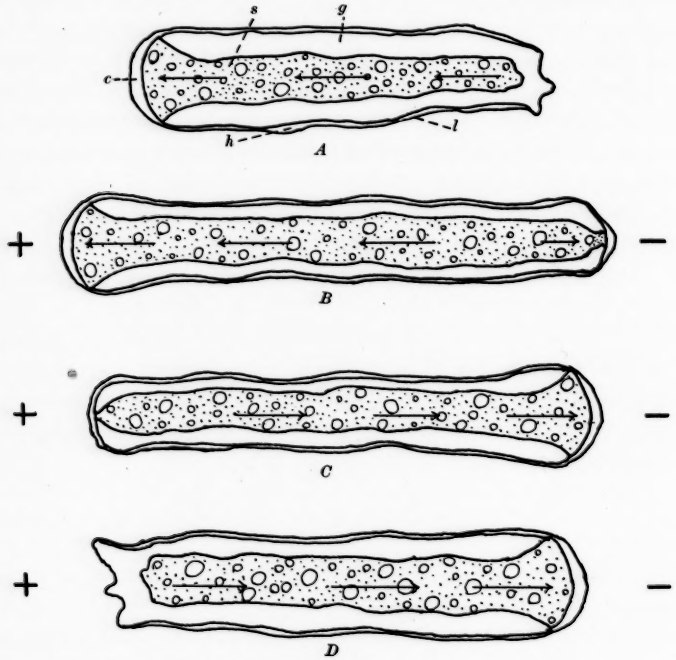

FIG. 11.—Sketches showing the changes produced by a galvanic current at the ends of an amoeba which is moving toward the anode. *A*, General relation of parts during normal locomotion; *B*, relation of parts soon after the current is made, showing reversal in the direction of flow of plasmasol at the cathodal end and continuation of the main flow toward the anode; *C*, relation of parts some time later, showing cessation of flow toward the anode and initiation of main flow toward the cathode; *D*, relation still later, showing the flow of the entire plasmasol toward the cathode. *g*, Plasmagel; *s*, plasmasol; *l*, plasmalemma; *c*, hyaline cap; *h*, hyaline layer; arrows, direction of flow of plasmasol; +, anode; −, cathode. Note that reversal in direction of flow may occur at the cathodal end before cessation of flow occurs at the anodal end.

direction of flow of the plasmasol. The following explanation is hypothetical, but it is in fair agreement with the observations.

In unstimulated specimens, the plasmasol throughout the whole

length of the organism flows toward the anterior end (Fig. 11, *A*) with solation occurring continuously at the posterior end and gelation occurring continuously at the anterior end. When the current is turned on, the thick layer of plasmagel at the posterior end, in specimens moving toward the anode, weakens and then breaks; now the plasmagel tube, being weak at both ends, contracts in the middle and forces plasmasol in both directions, thereby initiating the small reversed stream at the cathodal end and continuing the forward stream in other regions (Fig. 11, *B*). ¶If the current is weak, gelation or fusion soon closes the plasmagel tube at the cathodal end, after which the whole column of plasmasol again flows in the original direction. Now the organism gradually turns around and proceeds toward the cathode. If the current is strong, gelation at the cathodal end is not sufficient to completely close the tube with a thick layer of gel. The flow of plasmasol toward the cathode persists, and the flow of plasmasol toward the anode soon ceases (Fig. 11, *C*). Then the end of the plasmagel tube toward the anode closes, owing to gelation or contraction, after which the whole column of plasmasol flows toward the cathode (Fig. 11, *D*).

The results presented show clearly that the constant current provokes response in *Amoeba* by definite polar actions at the ends of the organism; that destruction (solation) of the plasmagel is produced almost immediately at the cathodal end; that then contractive and later disintegrative processes are initiated at the anodal end; and that these responses are directly dependent upon the strength of the current.

DISCUSSION

The results presented demonstrate, as stated above, that the response to electricity in *Amoeba* is due to some polar action of the current which causes destruction (solation) of the plasmagel at the cathodal surface of the organism. It now remains to consider the question as to how this solation is produced.

The only immediate effects of passage of current which might produce solation of the plasmagel at the cathodal surface are (1) movement of ions and (2) movement of water.

Solation due to the movement of ions might be brought about

by the action of positive ions which are carried along by the current and accumulated within the plasmagel on the side toward the cathode. Under these conditions, we may assume that the accumulation of positive ions in some way increases the porosity of the plasmagel, since water passes or is forced through it and collects under the plasmalemma, forming a clear blister; that either the continued action of the cations or this passage of water so weakens the plasmagel that it finally breaks down completely, forming a pseudopod which extends toward the cathode; and that the accumulation of negative ions within the plasmagel on the side toward the anode decreases the porosity of the plasmagel in that region, resulting in increase in its elastic strength. Solation at the cathodal end and increase in elastic strength at the anodal end would result in the flow of plasmasol toward the cathode.

Solation due to the movement of water through the plasmagel as a capillary membrane might be brought about by the electroendosmotic action of the constant current. It is known that the more solid phase in protoplasm is negatively charged with respect to the more liquid phase and that the plasmagel behaves as a fairly stable membrane. If we consider that the plasmagel acts as a capillary membrane and that it is coated with an electrical double layer, then the charge on the plasmagel is negative and that on the surrounding film of water is positive. An external electromotive force applied to such a system will cause a relative motion of the two layers. Since the negative charge is imbedded in the plasmagel and does not move, the layer of liquid, being positively charged and freely movable, will pass through the plasmagel toward the negative pole. Indeed, the first visible response to a current in amoebae attached to the substratum consists of the passage of water through the plasmagel. The subsequent movement of small and then large granules indicates clearly that the plasmagel gradually increases in porosity until it finally breaks down completely.

The data presented in this paper do not reveal precisely which of these two possible actions of the constant current is responsible for solation of the plasmagel on the cathodal side. On the basis of the foregoing discussion we should expect that either the action of accumulated positive ions or of the electroendosmotic movement of

water would account for it, and that either of these processes would show a definite and consistent relation to the strength and duration of the current.

Mast (1931) found that solation occurs in specimens subjected to alternating current, in which electroendosmosis cannot be involved. It is evident therefore that movement of water plays only a minor rôle, if any, in solation produced by alternating current and that it must be largely, if not entirely, due to differential accumulation of ions. If this is true for alternating current, it probably is also true for direct current.

SUMMARY

1. The rate of locomotion in monopodal specimens of *Amoeba proteus* under constant conditions is fairly constant over long periods of time.

2. The nature of the response to a galvanic current in *Amoeba* depends upon the direction of movement when the current is made and upon the strength of the current.

3. Specimens moving toward the cathode respond to sudden increase in current density by increase in rate of movement at the posterior end and decrease in rate at the anterior end, resulting in contraction of the organism. These changes in rate, which vary in magnitude with the strength of the current, persist only a few moments and then gradually disappear.

4. Specimens moving toward the cathode respond to prolonged passage of a constant weak current by increase in the rate of locomotion, which persists for several minutes. They respond to prolonged passage of a strong current by contraction, which is followed by disintegration of the organism beginning on the anodal side.

5. Specimens moving toward the anode respond to sudden increase in current density by reversal in the direction of protoplasmic flow at the cathodal end, followed about a second later by cessation of flow at the anodal end.

a) In weak currents, both are momentary, then movement is resumed in the original direction; in strong currents, both persist, and movement is continued in the opposite direction.

b) The time between the closing of the circuit and the response, reaction-time, varies nearly inversely with the square of the current

density; the time-intensity relation in this response is, however, more nearly in accord with the equation, $i\sqrt{t}-(xi)=K$, than with Nernst's equation, $i\sqrt{t}=K$, in which i is the intensity of the current and t its duration.

c) Both decrease in temperature and previous stimulation by the current cause increase in the reaction-time. This may be due to increase in viscosity of the cytoplasm.

6. Response to the constant electric current in *Amoeba proteus* is due to local decrease in the elastic strength of the plasmagel on the cathodal side of the organism.

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A PHYSIOLOGICAL STUDY OF THE INNERVATION OF THE HEART OF FISH EMBRYOS

(Seven figures)

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WITHIN the last quarter of a century a number of workers have been interested in studying the effects of various factors upon the cardiac rhythm of *Fundulus* embryos, giving little, if any, attention to whether the heart is beating myogenically or controlled by the extrinsic nerves. It is well known that the embryonic hearts of various animals establish rhythmic contractions before contact is made with the nervous system. However, after innervation, the heart rhythm and amplitude is more or less regulated by the controlling action of the extrinsic nerves. In studying the effect of any factor on the heartbeat, it is not only desirable, but may prove to be essential, to know whether or not the heart is under the control of the extrinsic nerves and, if so, when this contact is made. An innervated heart is an entirely different physiological mechanism than a heart not controlled by the nerves. In the case of an innervated heart, the rhythm and amplitude of the beat may be altered by the action of some environmental factor either on the extrinsic nerves or on the cardiac muscle and nodal tissue. The possibility of the former effect is automatically ruled out in a myogenic heart.

A study of the effect of caffeine upon the cardiac rhythm of chick embryos (Brinley, 1932) shows that the primary action of the drug is on the vagi supplying the heart, with a possible secondary effect on the auricular-ventricular bundle of His. There appears to be no noticeable effect upon the cardiac muscle. It occurred to the writer, while conducting this investigation, that this method may be used to determine the time of innervation of the hearts of fish embryos. The work reported in the present paper was conducted on three species of fresh-water fish at the University of Michigan Biological

Station in 1930 and on *Fundulus heteroclitus* at Woods Hole in 1931. Abstracts appeared in the *Anatomical Record*, December, 1930, and December, 1931.

MATERIAL

Four species of fish were used in these experiments: the common yellow perch (*Perca flavescens*), the log-perch (*Percina caprodes*), straw-colored shiner (*Notropus deliciosus*), and the marine minnow (*Fundulus heteroclitus*). The eggs of these fishes proved to be excellent material for studying the action of the heart and circulation. The chorion and yolk are transparent, and development can be easily followed without any disturbance to the embryo. The heart action of the fresh-water fishes can be easily seen, as the chromatophores do not appear until after hatching. The large, dark chromatophores in *Fundulus* appear about the third day after fertilization, and at times are quite a hindrance to proper observation of the embryo. The egg shells of all species can be punctured or completely removed after the heart starts to beat, without any injury to the embryo. Owing to the fact that the fresh-water species may not be as well known as the marine minnow, the reader is referred to a paper by Brinley and Creaser (1931) for a brief description and general notes on the distribution of the species used.

METHOD

Eggs were stripped from the females (except perch) and fertilized in Syracuse watch glasses, Petri dishes, or finger bowls, and allowed to develop. Eggs were removed from the developing dishes at various intervals after pulsations of the heart had started, and were placed in watch glasses containing caffeine solution (Merck U.S.P.) in concentrations ranging from M/100 to M/1,000. The solutions were made up in pond or sea water, depending on whether the fish were fresh- or salt-water species. The majority of the experiments were conducted in triplicates. The egg shells were either completely removed or punctured with fine glass needles. The time required for 50 cardiac contractions was taken with a stop watch, and the number of beats per minute computed. Any irregularity or variation in the amplitude and rate of beat was carefully noted. The temperature of the laboratory at the time the experiments were

conducted varied from 21° to 26°, but the temperature did not change more than 1° during any experiment. No attempt was made to hold the eggs at a constant temperature during development; therefore, the age in hours or days is only roughly comparative. All solutions were slightly alkaline, having a pH about 8.2 or 8.4, which was the same pH as the water in which the eggs were developing.

RESULTS

Perch (Perca flavescens).—Perch eggs were obtained in April from a state fish hatchery in Hastings, Michigan. The eggs were collected in ponds after they had been naturally spawned, and were placed in the hatchery. Therefore, the exact time of fertilization was unknown. The eggs were kept in the laboratory in large crystallizing dishes, and the rate of development noted. Cardiac contractions started on the fourth day after the eggs were obtained from the hatchery, or about 6 or 7 days after fertilization. The cardiac contractions were slow and irregular during the first day, but on the second and succeeding days the rate became more uniform and constant at about 125 beats per minute. Circulation was pronounced in the large blood vessels on the second day after pulsations commenced.

At various intervals after the starting of the cardiac contractions, the shells were either removed from a number of the eggs or punctured with a needle and the embryos placed in caffeine solution. The time required for 50 heartbeats was taken with a stop watch, and the number of beats per minute computed. Caffeine in concentrations varying from M/100 to M/1,000 did not seem to affect the rate or amplitude of the beat until about 50–60 hours after the heart had commenced to beat (Fig 1). On the third day after the cardiac contractions started, caffeine produced a decided irregularity in the cardiac rhythm within 10–20 minutes after immersion in the drug (Fig. 1, No. 4). The initial effect of the drug on the heart is shown by a complete stoppage for a few seconds, and then the beat was resumed at a normal or slightly accelerated rate. In many cases the time required for 50 beats, or the number of beats per minute, does not give a true index of the conditions, owing to the fact that the pauses in the rhythm are often followed by an acceleration in

the time for 50 beats, or the number of beats per minute, remains constant. However, the irregularity in the rate is so marked that it cannot escape observation. The cardiac rhythm, after the initial effect, again becomes regular; but the rate is much slower. In many

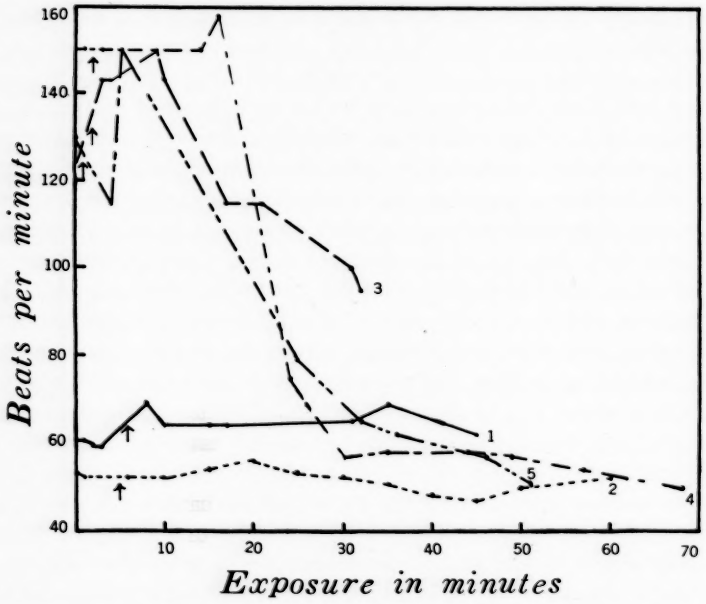

FIG. 1.—The effect of caffeine on the cardiac rhythm of perch embryos, *Perca flavescens*. The arrows indicate the time the embryos were placed in M/100 caffeine solution. The index to the graph figures, which denote the time in hours after cardiac pulsations started, is as follows: *1*, 1 hour; *2*, 3 hours; *3*, 32 hours; *4*, 53 hours; *5*, 54 hours.

cases, after the initial action of caffeine, the auricles continued to beat at a more or less constant rate but the ventricles would skip beats at rather definite intervals, approaching the heart-block condition observed in chick embryos (Brinley, 1932).

Log-perch (*Percina caprodes*).—The adult log-perch were seined along the sandy shores of Douglass Lake in northern Michigan. The females were immediately stripped, and the eggs placed in

Syracuse watch glasses. Eggs from different individuals were not placed in the same dish. Two or three males were stripped, and the concentrated sperm was mixed with the eggs in each dish. Water was added within 2 or 3 minutes, and the eggs were allowed to develop in the laboratory as previously described. The cardiac contraction started on the second or third day; at first the beat was slow and irregular, but after about 70 hours the rate became uniform at 115–20 beats per minute. At various intervals after cardiac contractions started, eggs were removed from the developing dishes and transferred to M/100 caffeine solution, and the time required for 50 beats was taken with a stop watch. Caffeine does not seem to affect the rate or amplitude of the beat until about 75–85 hours after fertilization, or about 60 hours after pulsations start (Fig. 2). In some experiments (Fig. 2, Nos. 3 and 5), the heart-rate seems to be slightly accelerated after immersion in caffeine; but as this reaction is not consistent in all experiments, it is probably not of great significance. After about 77 hours of development, the heart-rate of the embryo, after immersion in the drug for 20 minutes, becomes very irregular, and long pauses of several seconds' duration occur at rather frequent intervals. The rate after a longer immersion again becomes constant but much slower. The same reaction occurs in newly hatched larvae.

Straw-colored minnow (*Notropus deliciosus*).—The adults of the straw-colored minnow were collected at night in Douglass Lake and kept in a live-box immersed in the lake until the following morning. The individuals were then stripped, and the eggs fertilized. The development of the eggs in this species is very rapid at room temperature. The cardiac contractions start on the second day, and hatching takes place about 72 hours after fertilization. Eggs were immersed in M/100 caffeine solution as soon as the heart started to beat; and within a few minutes the rhythm became very irregular, with long frequent pauses, followed by a series of beats in rapid succession (Fig. 3). Thus the embryos of *Notropus* differ from the other species in that caffeine alters the cardiac rhythm as soon as the heart starts to beat. This may be explained by the rapid development of this species.

Fundulus heteroclitus.—*Fundulus* were stripped and fertilized in the usual manner, and the eggs were immersed in caffeine solution

at various intervals after the heart started to beat. Caffeine in M/100 concentration did not produce any decided effect upon the rate or amplitude of the beat until the tenth day of development (Fig. 4, No. 4). After that time the rhythm became irregular and the heart behaved in the same manner as described in the other species.

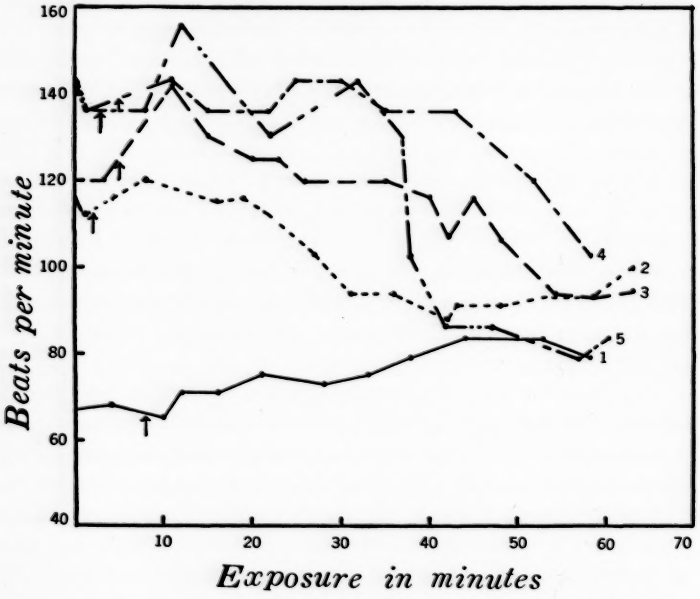

FIG. 2.—The effect of caffeine on the cardiac rhythm of log-perch embryos, *Percina caprodes*. The arrows indicate the time the embryos were placed in M/100 caffeine. The index to the graph figures, which indicate the time in hours after fertilization, is as follows: *1*, 67 hours; *2*, 75 hours; *3*, 77 hours; *4*, 82 hours; *5*, 84 hours.

The stage of development in which the heart responds to treatment is after the organ has flexed, the mouth cavity has broken through, and the mandibles become movable, which is often less than a day prior to hatching. The newly hatched larvae respond to caffeine in the same manner as the embryos within the eggs.

CONTINUOUS IMMERSION

A series of experiments were conducted on the effect of dilute solutions of caffeine upon the development of *Fundulus*. The eggs

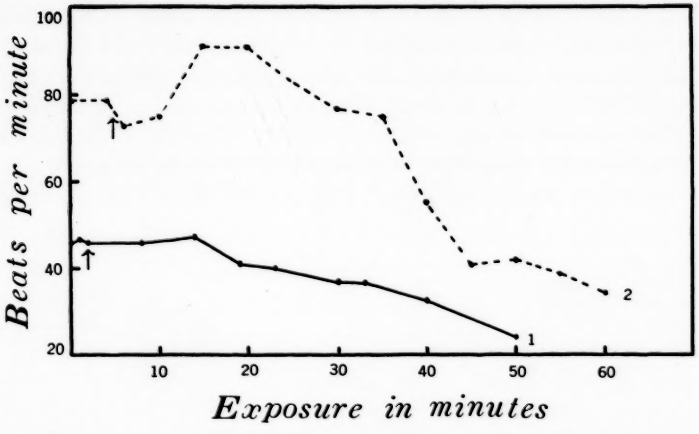

FIG. 3.—The effect of caffeine on the cardiac rhythm of *Notropus deliciosus*. The arrows indicate the time the embryos were placed in M/100 caffeine. The index to the graph figures, which indicate the time in hours after fertilization, is as follows: *1*, 42 hours; *2*, 43 hours.

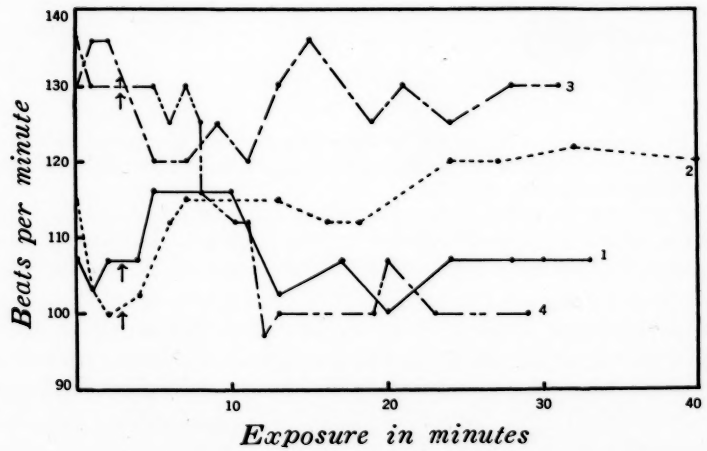

FIG. 4.—The effect of caffeine on the cardiac rhythm of *Fundulus heteroclitus* embryos. The arrows indicate the time the embryos were placed in M/100 caffeine. The index to the graph figures, which denote the time in days after fertilization, is as follows: *1*, 3 days; *2*, 5 days; *3*, 9 days; *4*, 10 days.

were placed, continuously from fertilization until death, in M/500 and M/1,000 caffeine solution. As accurately as could be determined by gross examination with the microscope, the larvae developed normally at the same rate as the controls until the heart commenced to beat. After that time, the heart continued to pulsate but developed into a long, straight tube with slight enlargements at the

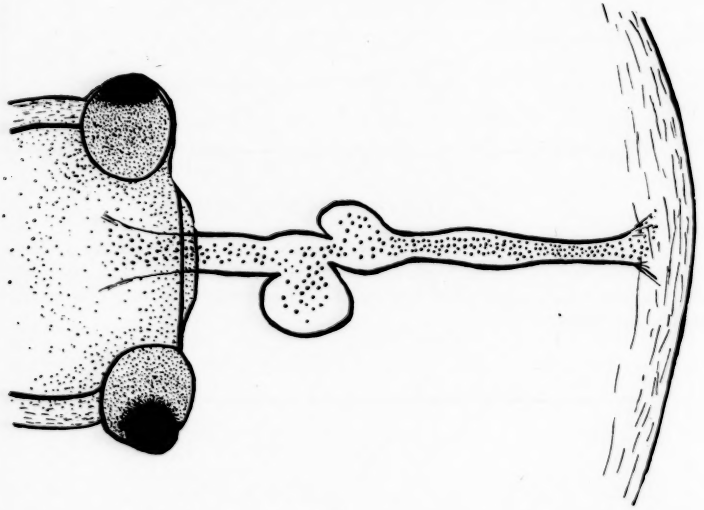

FIG. 5.—A sketch of the heart of *Fundulus* embryos shortly after pulsations started in embryos which had been continuously immersed in dilute solutions of caffeine from the time of fertilization.

sinus, auricular, and ventricular regions (Fig. 5). The cardiac contractions could be easily followed, as the wave of contraction was much slower than in the controls. The contraction would start as a peristaltic wave in the sinus or at the junction of the great veins with the sinus, and slowly travel over the auricle to the ventricle. The first wave always completed its course before the second one started. The heart seldom flexed as in the normal embryo (Fig. 6). Circulation did not occur in the arteries and veins, but the corpuscles could be seen to ebb and flow in the heart as the contraction wave passed through that structure. Finally the heart would stretch to

such a degree that the walls would come together (Fig. 7). The corpuscles could be seen within the tube, but they did not move as the heart contracted. The heart would continue to pulsate in this condition for several days, long after disintegration of the embryo, especially the head, had started. The heart would beat for several days after the time of hatching of the controls, but the treated eggs never hatched in M/500 or M/1,000 caffeine. Eggs immersed in

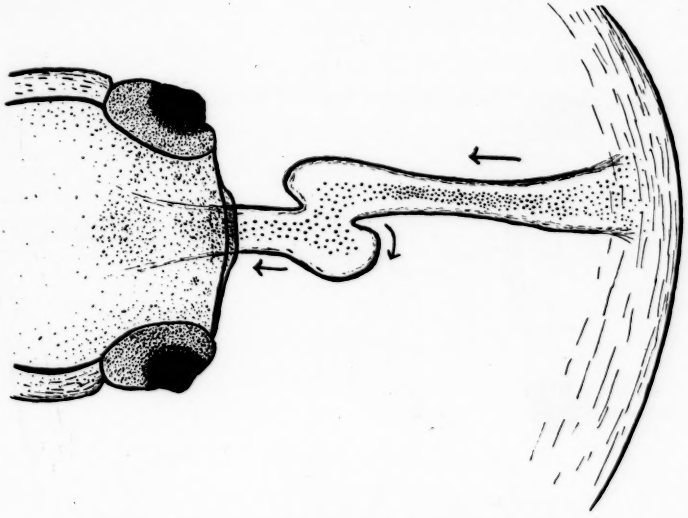

FIG. 6.—A normal embryo of the same age as in Figure 5

M/10,000 caffeine solution developed into normal larvae, but the time of hatching was delayed.

CONCLUSIONS

It is apparent from the results of the foregoing experiments, on the effect of caffeine upon the heartbeat of fish embryos, that at a more or less definite stage of development, the rate and rhythm of the beat is altered in a very characteristic manner by the addition of caffeine. The initial reaction to the drug is noticeable by frequent pauses and a decided irregularity in the rhythm. This condition changes in a few minutes to a constant rate which is much slower

than the normal. This secondary effect persists for several hours, until death of the embryo. This irregularity in the rate seems to be typical vagus stimulation, as is found in other types of animals. The author has previously shown (1932) that the effect of caffeine on the embryonic chick heart after innervation by the extrinsic nerves is decidedly different from that on the prevagus heart, and it was concluded that the drug depresses the heart-rate by its action on the nervous system and that it also may reduce the conducting

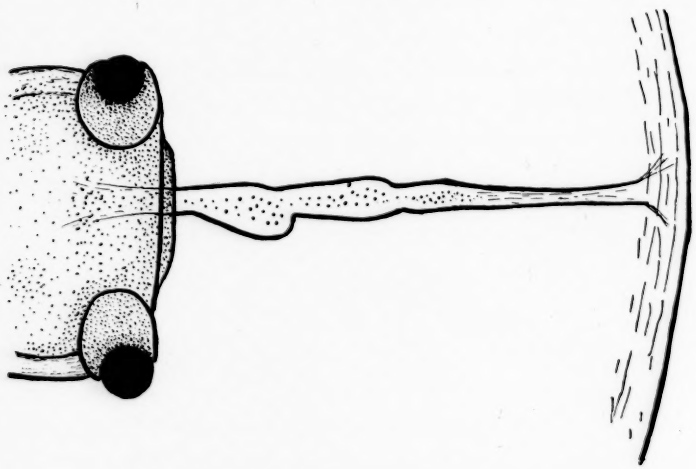

FIG. 7.—The same embryo as in Figure 5 but 2 days later

power of the bundle of His. It is believed that the stage in development of the fish embryos which shows the initial effect of the drug is the time that the vagus connects with the heart. No sympathetic innervation has been demonstrated in the fish embryo. The time of innervation of the heart depends on the specific nature of the fish. Various attempts have been made to correlate the time of innervation with some developmental condition of the embryo, but only partial success has thus far been obtained. In the perch and logperch the nerves seem to make contact with the heart about the time the tube has flexed to form the chambered heart. In the case of *Notropus* the heart shows the effect of the drug as soon as con-

tractions start, before the heart has flexed. *Fundulus* does not show the effect of the drug until development is much more advanced than in the fresh-water species. In *Fundulus* the vagus apparently makes its connection with the heart about the time the mouth has cut through and the mandibles become movable. This stage seems to correspond with stage 5 in Armstrong's recent work. During this stage, Armstrong (1931) states that the vagus completes its innervation.

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HIBERNATION AND DIAPAUSE IN CERTAIN ORTHOPTERA. II. RESPONSE TO TEMPERATURE DURING HIBERNATION AND DIAPAUSE

(Seven figures)

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QUESTIONS of hibernation and diapause among animals, in addition to their inherent interest as such, are of much concern to the physiologist, since their analyses may give information or throw light upon the fundamental actions and reactions of the cells of an organism as well as the relations between the organism and its environment. Such fundamental problems as the causes of cessation of development and differentiation, of inhibition of mitosis, and other cellular activities have occupied the minds of biologists for some time. Recent extensive literature, reviews, and discussions by Uvarov (1931) and others contain rather complete surveys on hibernation, diapause, etc., in lower animals, and particularly in insects, so that no attempt will be made here to duplicate such effort. The reader is referred to these monographs, where rather complete bibliographies and detailed data may be found.

In the present paper some phases of the physiology of hibernation and diapause in certain Orthoptera (grasshoppers) will be discussed. In a previous communication (Bodine, 1923) certain aspects of this problem have been presented. The present data are concerned almost exclusively with questions of hibernation and diapause in the nymphs of *Chortophaga viridifasciata* and in the eggs of *Melanoplus differentialis*. The life-cycles of these two species are sufficiently different to justify a brief review of them before going farther. *Chortophaga viridifasciata*, in the vicinity of Iowa City, lays its eggs in the soil in late spring or early summer; and these hatch in late summer and early fall. The nymphs, reaching the second or third instars, hibernate, and in spring grow and become mature in early summer. *Melanoplus differentialis*, on the other hand, normally lays its eggs in late fall, the eggs remaining in the ground during the winter and

hatching in early summer. The nymphs grow and become mature during the summer. Low temperatures or winter are thus passed through in quite different stages in the two species—the nymph in the case of *C. viridifasciata* and the egg in *M. differentialis*. The specific problems to be dealt with here have to do with an attempted analysis of the physiological reactions to temperature in the two organisms during their hibernation, or rest periods.

It would be well, however, to point out the manner in which the two terms "hibernation" and "diapause" are to be used in what follows. The term "hibernation" refers to the normal living of the organism out-of-doors during the winter or periods of low temperature. "Diapause," as originally used by Henneguy (1904), refers to a cessation of activity, embryonic or otherwise, not necessarily conditioned by temperature.

Animals and eggs used in these experiments were normal out-of-door individuals as well as those laboratory reared and controlled. Oxygen determinations were carried out in constant-temperature baths, using the modified Krogh manometer (Bodine, 1929). Techniques employed were essentially similar to those used in previous work, to which the reader is referred for further details (Bodine, 1929).

HIBERNATION IN NYMPHS OF CHORTOPHAGA VIRIDIFASCIATA

In a former publication (Bodine, 1923) it was pointed out that prior to and during hibernation, nymphs of *C. viridifasciata* contained relatively low and constant amounts of water and that these low water contents were undoubtedly closely associated with the animal's ability to withstand exposures to low temperatures. It was also noted that recovery from hibernation and subsequent growth were, to a great extent, associated with a readjustment and increase in the amount of water the animal contained. In order to show that no fundamental differences other than low water content and inability to grow are involved in hibernation in these forms, extensive experiments have been carried out in measuring the rates of oxygen consumption in nymphs entering, during, and recovering from normal out-of-door hibernation, as well as in nymphs raised and kept only at higher temperatures (20°–22° C.) throughout their

life-cycle. The effects of different temperatures on the rates of oxygen consumption in these animals have also been studied. Figure 1, giving typical graphs, shows that no fundamental differences in rates of oxygen consumption at different temperatures for different animals seem to exist. Hibernating animals, or those with relatively constant low amounts of water, respond as do growing non-hibernating nymphs. As a matter of fact, the effects of temperature on the rates of oxygen consumption for *C. viridifasciata* resemble closely the results obtained by Rogers (1929) for the nymphs of *M. differentialis*. Figure 2, in which the rates of oxygen consumption of *C. viridifasciata* are plotted along with results of similar experiments by Rogers (1929) for *M. differentialis*, shows that nothing in the reactions of *C. viridifasciata* would indicate any difference in individuals hibernating or non-hibernating. The same general conclusions reached by Rogers (1929) as to the effect of different temperatures on the oxygen exchange of *M. differentialis* can be applied to *C. viridifasciata*. It is, perhaps, of some interest to add that often, in raising *C. viridifasciata* at constant high temperatures, one notes individuals growing at a slower rate than others hatched from the same pod. That these slow-growing animals are not different in their temperature responses from others is shown in Figure 1.

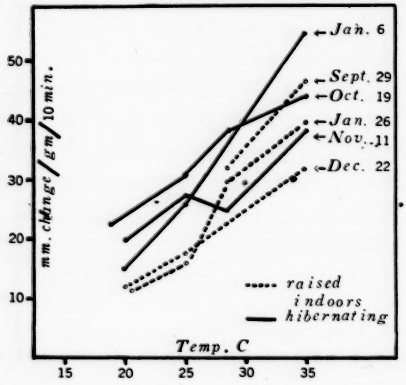

FIG. 1.—Typical effects of temperature on rates of oxygen consumption for hibernating out-of-door nymphs as well as for nymphs raised at constant high temperatures. Ordinates, millimeter change of manometer per gram per 10 minutes—same manometer used throughout, and conversion factor for oxygen values same for all readings. Abscissae, temperature. Dates indicate time of year experiment was carried out. For further explanation see text.

Hibernation in *C. viridifasciata* nymphs seems, therefore, to be conditioned largely by low temperatures; and no indication of an inherent rest or diapause independent of temperature seems evident.

HIBERNATION AND DIAPAUSE IN MELANOPLUS DIFFERENTIALIS

As pointed out above, eggs of this species are normally laid in the soil late in the summer and remain there until the following spring and early summer, when they hatch. Rather intensive morphological and physiological studies, under controlled temperature conditions, have shown that eggs of this species are extremely complex and have an interesting range of peculiarities. A timed temperature-cytological-morphological-physiological picture of the development of the animal is now available whereby fairly normal conditions can be ascertained (Bodine, 1929; Slifer, 1932*a*, 1932*b*).

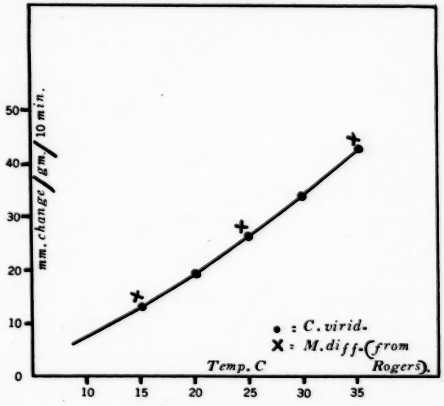

FIG. 2.—Average effects of temperature on rates of oxygen consumption for hibernating and non-hibernating nymphs of *Chortophaga viridifasciata*, as well as results from Rogers for nymphs of *Melanoplus differentialis*. Rest of description same as Figure 1.

Slifer (1932*a*) has shown that females of *M. differentialis* lay two types of eggs, one exhibiting a diapause, the other showing none. The eggs of the first type (with diapause) develop at 25° C. to the 3-week stage, at which time they cease development and enter a true diapause. The eggs of the second type (without diapause) develop at 25° C. in a regular and more or less uniform manner from time of laying to hatching. It is the diapause egg with which this discussion is chiefly concerned, and some physiological peculiarities of this type of egg in respect to its temperature reactions are presented.

If rates of oxygen consumption of diapause-type eggs, kept at constant high temperatures, are followed from time of laying to hatching, a type of curve is produced as shown in Figure 3. An inspection of this figure shows a regular and fairly rapid rising rate of oxygen consumption during the first 3 weeks of development, which

is followed by an equally rapid decrease in rate to a minimum value. This relatively low rate of oxygen consumption continues over a period of time (diapause), at the end of which an increase in rate occurs and culminates in the hatching of the egg. Such a curve is typical for all eggs of this type (with diapause) when kept at constant high temperatures above developmental zero. Morphologically interpreted, the animal grows at a normal rate until the 3-week

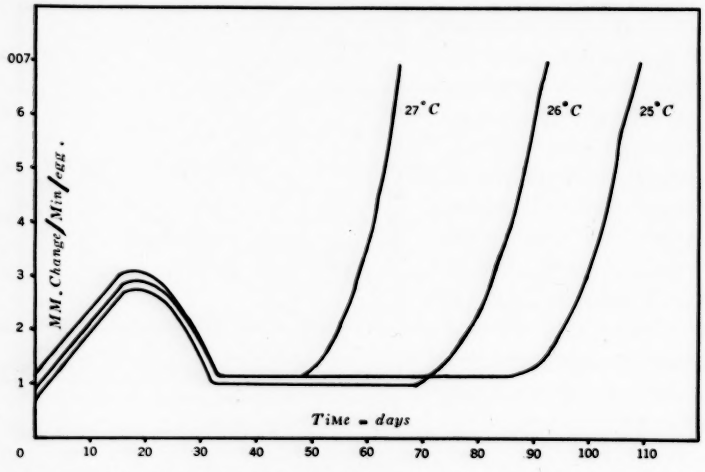

FIG. 3.—Rates of oxygen consumption for diapause-type eggs of *Melanoplus differentialis* at constant temperatures from time of laying to hatching. For further description see text.

stage is reached (Slifer, 1932*a*), when diapause or cessation of development occurs. During this time (diapause) no mitotic spindles are found (Slifer, 1931), and development seems completely arrested. The closest sort of morphological and physiological correlations are apparent in following through the development of such eggs.

Diapause, it will be noted, is, for its occurrence, quite independent of temperature, since it invariably occurs in this type of egg at all high temperatures (above developmental zero) examined. That its length is influenced by temperature will be noted from an inspection of Figure 3, obtained for eggs kept at different constant tempera-

tures, as well as by reference to work by Parker (1930) on a closely related species.

When eggs in diapause are subjected to different high temperatures and rates of oxygen consumption are determined, rather regular increases in rates with increases in temperature are found (Fig. 4). For increases of 10° C., rates of oxygen consumption are doubled or trebled, as is the case for normal non-diapause eggs. Diapause

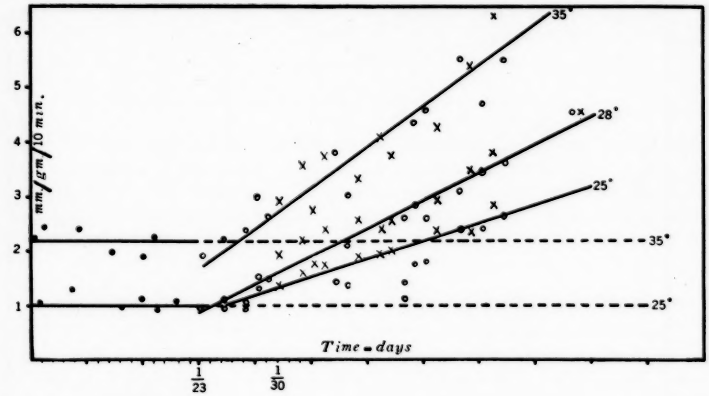

FIG. 4.—Rates of oxygen consumption at different temperatures for eggs in diapause, and after destruction of diapause factors by exposure to low temperature (2° C.). Dots (•), eggs in diapause. Circles (○), eggs in diapause subjected to 2° C. for 2 days to destroy diapause factors, then returned and kept at room temperature (20° C.). Crosses (×), eggs in diapause subjected to 2° C. for 10 days to destroy diapause factors, then returned and kept at room temperature (20° C.). Dotted lines represent possible rates of oxygen consumption for diapause eggs at temperatures indicated. Explanations as in Figure 1.

eggs, while in diapause, give much lower values for rates of oxygen consumption than growing non-diapause eggs in correspondingly similar morphological stages of development (Figs. 3, 4, and 5). Low rates of oxygen consumption are invariably associated with the diapause condition, and these seem to be quite independent of the surrounding temperature, except as above noted.

If diapause-type eggs are put at low temperatures (10°–0° C.) at any time before or after the 3-week stage, diapause can be prevented or destroyed. Figure 5 shows graphically the rates of oxygen con-

sumption for freshly laid eggs kept at 10° C. for varying periods and then subsequently put at higher constant temperatures and allowed to develop. It will be noted that rates of oxygen consumption in these eggs at higher temperatures are similar to those for developing non-diapause eggs (Fig. 7). It will be further noted that the same result can be obtained, i.e., no diapause, when eggs are subjected to low temperatures anytime before the 3-week stage (Fig. 6). If the

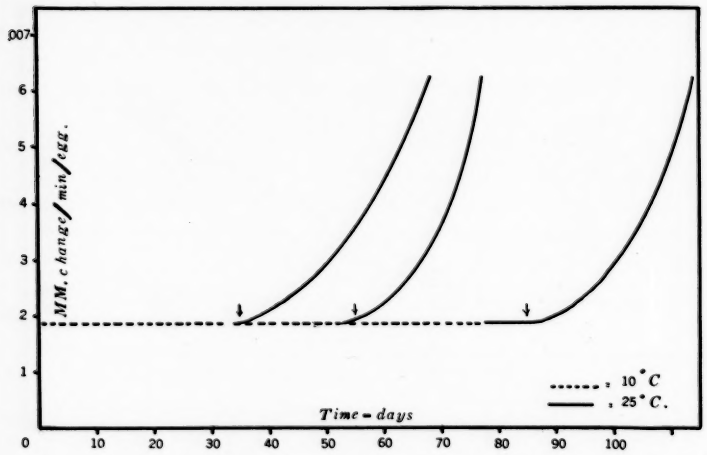

FIG. 5.—Rates of oxygen consumption for eggs put and kept at 10° C. from day of laying to various periods, at which points they are transferred and kept at constant high temperature (25° C.) till hatching. Explanations as in Figure 1. For further description see text.

egg has reached the 3-week stage (diapause) at higher temperatures, diapause can readily be broken up or destroyed by subjecting the egg to low temperature (Bodine, 1929; Parker, 1930; Slifer, 1932*a*, 1932*b*). Figures 4, 5, and 6 show graphically the effect of different temperatures upon the rates of oxygen consumption of eggs at different stages of development in which the diapause factor has been destroyed by previous exposure to low temperatures normally, out-of-doors, and experimentally in the laboratory. From an inspection of these curves it can readily be seen that the rates of oxygen consumption for eggs thus treated are considerably higher than for eggs in

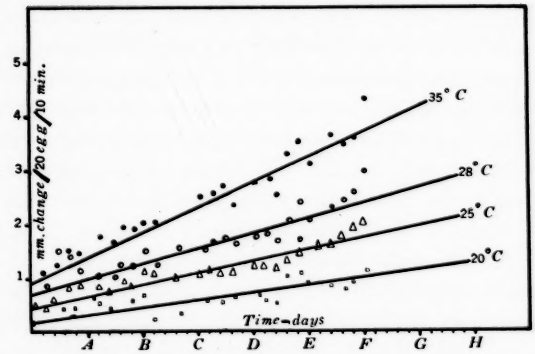

FIG. 6.—Rates of oxygen consumption at different temperatures for out-of-door eggs, hibernating at the 14-day morphological stage, when put and kept at room temperature (20° C.) till hatched. Initial readings are for the day eggs were brought indoors. Space between letters on abscissae indicate periods of 5 days. For further description see text.

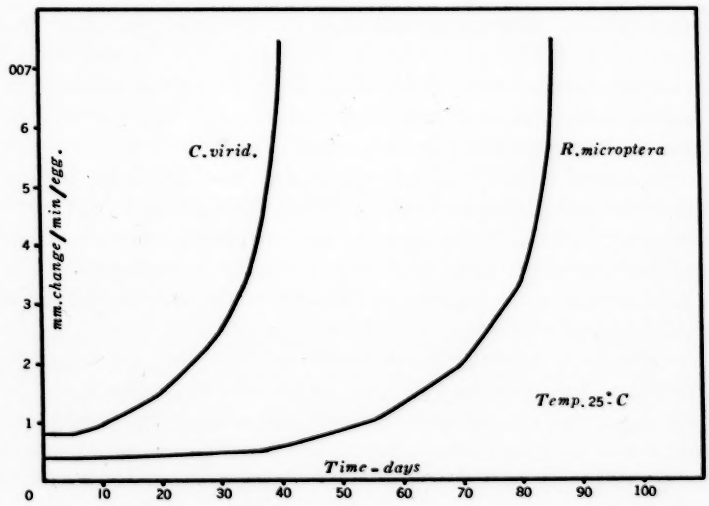

FIG. 7.—Rates of oxygen consumption for eggs of *Chortophaga viridifasciata* and *Romalea microptera* kept at constant temperature of 25° C. from laying till hatching. For further description see text.

diapause. The relative effects of different temperatures, however, are somewhat similar to those for the eggs in diapause, since increases in rates for increases of 10° C. are of the same general magnitude i.e., 2 to 3. It has also been found that for eggs in which the diapause factors have been destroyed by appropriate exposure to low temperature, no marked differences in response to different temperatures from the non-diapause-type egg in corresponding morphological stages of development exist.

That eggs of *M. differentialis* are quite different and distinct from those of other species can be seen by inspection of Figure 7, which shows graphically the rates of oxygen consumption for eggs of *C. viridifasciata*, and *Romalea microptera*, which are normally never subjected to anything like the extremes of temperature as are eggs of the former species. Recent chemical investigations on the nature of the fats contained in eggs of different species of grasshoppers by Slifer (1932*b*) also indicate marked specific differences.

DISCUSSION

Questions of hibernation and diapause in Orthoptera have been of interest to the writer in view of the possibilities, offered through their study, of obtaining some clue or insight into more fundamental cellular phenomena, such as mitosis, cessation or inhibition of development, etc. Since there is now available a more or less complete morphological, cytological, and physiological picture of the embryo under controlled temperature conditions throughout its entire developmental period, it should be of much practical interest to study the various factors influencing these normal developmental cycles.

Hibernation in the nymph of *C. viridifasciata* and in the egg of *M. differentialis* present rather interesting variations. Low temperatures, for both, apparently are associated with low and relatively constant water contents. This low water content undoubtedly enables the animals to withstand harmful freezing effects of low temperatures. Diapause in the egg of *M. differentialis* seems to be of an entirely different nature from hibernation, inasmuch as it is relatively little affected by temperatures above developmental zero. During the diapause period, however, water relations remain low, very much the same as in hibernation. The occurrence of diapause, as pointed

out above, seems independent of temperature; while its length or duration is, to a great extent, conditioned by temperature and perhaps by moisture (Parker, 1930). Hibernation in the case of the egg of *M. differentialis* seems largely a question of the effects of low temperature on diapause factors and growth factors, as more fully discussed in the following paper. In subsequent communications detailed data on various factors involved in the production and destruction of diapause are to be presented.

SUMMARY AND CONCLUSIONS

1. Comparisons between certain physiological reactions of hibernation in nymphs of *Chortophaga viridifasciata* and of diapause in eggs of *Melanoplus differentialis* have been presented.

2. Hibernation in nymphs of *C. viridifasciata* is almost solely dependent on temperature. Responses of hibernating animals to different temperatures are of same character as responses of non-hibernating animals.

3. Rates of oxygen consumption and effects of temperature on same for hibernating and non-hibernating nymphs of *C. viridifasciata* are presented.

4. Diapause in eggs of *M. differentialis* is, within limits, independent of temperature (above developmental zero) for its occurrence, but relatively dependent on temperatures (above developmental zero) for its duration.

5. Low temperatures (10°–0° C.) destroy the diapause factors in eggs of *M. differentialis*.

6. Effects of temperature on rates of oxygen consumption of diapause eggs, as well as for eggs with no diapause, are presented.

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HIBERNATION AND DIAPAUSE IN CERTAIN ORTHOPTERA. III. DIAPAUSE—A THEORY OF ITS MECHANISM

(Four figures)

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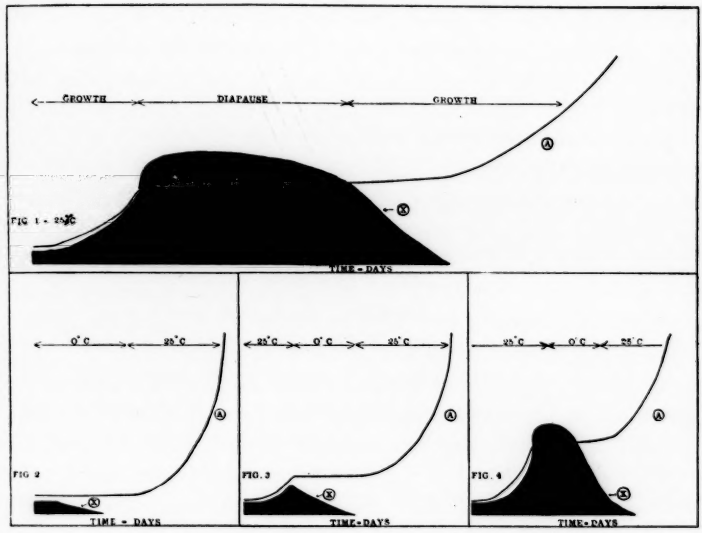
EXISTING data on the effects of temperature, etc., on the embryonic development of grasshoppers are of much interest; but when considered in view of more recent knowledge concerning embryonic diapause, they can be shown to be of little value except as starting-points for more fundamental investigations (Bodine, 1925; Uvarov, 1928; Parker, 1930.) To satisfactorily analyze problems on the physiology of development in these forms, it becomes more and more evident that studies must be carried out on single eggs, the morphological, physiological, and genetical histories of which are accurately known. The closest possible correlations between the morphology and physiology of the egg must be made before an appreciation of either of the two can be fully understood. From existing and recent data, however, it is possible to show something of the fundamental working of diapause and other factors during embryonic development. The following discussion, an interpretation based upon existing data and that more recently acquired, is an attempt to give a possible explanation of various phenomena connected with diapause occurring during the embryonic development of certain grasshoppers. Existing theories as to causes of diapause will not be discussed at this time, and the reader is referred to the recent monograph of Uvarov (1931) for complete bibliography of same.

As pointed out in the preceding article (Bodine, 1932), two main classes of eggs in *Melanoplus differentialis* seem to be produced: (1) a non-diapause type, in which no embryonic diapause can be demonstrated, the embryo developing at a uniform rate at constant high temperature (above developmental zero) from laying until hatching; and (2) a diapause type, in which a marked cessation of embryonic

development occurs (diapause) in eggs kept at constant high temperature above developmental zero. The present discussion deals entirely with the second type of egg, inasmuch as there seems to be nothing unusual in the development of the non-diapause type.

All diapause-type eggs, at the time of laying, seem to contain certain factors for the production of an arrest in development which manifests itself in eggs, kept at constant high temperature (above developmental zero), at a definite morphological stage. For *M. differentialis*, this stage, as shown by Slifer (1932), is designated as the 3-week stage for eggs kept constantly at 25° C. These so-called "diapause factors" can be thought of in several ways, either as chemical, physico-chemical, or, perhaps, physiological-genetic factors, present in more or less constant amounts in the egg at the time of laying. When freshly laid eggs are put and kept at constant temperatures (above developmental zero), normal development, characteristic in rate for the species and temperature, takes place (Bodine, 1925; Parker, 1930; and others). No apparent effect of the diapause factors, which, for sake of convenience, we shall term "*x*," can be noted on the rate of development of the egg for the first 3 weeks in the case of *M. differentialis* (Fig. 1) (Slifer, 1932). Comparisons between rates of development of non-diapause and diapause eggs show them to be quite similar in respect to developmental rates at constant temperatures (above developmental zero) for the first 3 weeks. In the diapause eggs of *M. differentialis*, diapause factors (*x*), during the course of the 3 weeks' development, increase either in potency or amount until they reach a certain threshold value, at which time they completely inhibit all developmental processes (Fig. 1). Otherwise expressed, developmental factors inherent in the embryo and termed "*a*," are inhibited by the now potent diapause factors (*x*). That this reaction is more or less of the "all or none type" is strikingly shown by the fact that no mitotic activity or other evidences of growth or differentiation are noted during the period and that occurrence of diapause takes place at always the same stage in development (Slifer, 1932). Marked changes in the rates of oxygen consumption have also been shown to occur at this stage (Bodine, 1929). If the eggs in this condition (diapause factors [*x*] causing complete inhibition of developmental factors [*a*]) are allowed to remain at constant high temperatures, hatching occurs only after relatively long



FIGS. 1–4

FIG. 1.—Shows, schematically, effect of constant high temperature (25° C.) on rate of development (*a*) and on rate of formation of diapause factor (*x*) in eggs of certain grasshoppers from time of laying to hatching. Abscissa represents time in days; ordinates, relative changes in rates or amounts. Solid, continuous line (*a*) represents growth-rates; solid black shaded region represents growth of diapause factor (*x*). For further explanation see text.

FIG. 2.—Destructive effects of low temperature (0° C.) on diapause factor (*x*); and also effects of low temperature (0° C.), as well as effects of subsequent exposure to high temperature (25° C.), on rates of growth in freshly laid eggs. Abscissa and ordinates as in Figure 1. Solid continuous line (*a*) represents growth-rates; solid black shaded region represents destruction by low temperature of diapause factor (*x*). No growth or development in embryo takes place during exposure to (0° C.). Marked and relatively constant growth results after exposure to constant high temperature of 25° C. No effects on diapause factor (*x*) are to be noted. For further description see text.

FIG. 3.—Effect on eggs on initial exposure to high temperature (25° C.), followed by period of exposure to low temperature (0° C.) with destruction or inhibition of diapause factor (*x*) and cessation of growth (*a*) due to low temperature (0° C.), and subsequent exposure to high temperature accomplished by normal development culminating in hatching. Explanations same as in Figure 2. For further explanation see text.

FIG. 4.—Effect on eggs of initial exposure to high temperature (25 °C.) until production of true diapause by diapause factor (*x*), followed by period of exposure to low temperature (0° C.) with destruction of diapause factor (*x*) to below threshold value and subsequent exposure to higher temperature (25° C.), accompanied by resumption of normal development culminating in hatching. Explanation same as in Figure 2. For further explanation see text.

periods of time and in more or less sporadic fashion. Eggs from the same pod hatch over rather long periods of time (Bodine, 1925; Parker, 1930; Slifer, 1932). The diapause factors (x), once they have built up a concentration or potency above a threshold value sufficient to cause complete inhibition of developmental factors (a), when kept at constant high temperatures gradually lose their potency or strength and after different periods of time, depending to a great extent upon the degree of temperature at which the eggs are kept, become so weakened or destroyed (falling below threshold value) that the developmental factors (a) again gain the upper hand and normal development ensues (Fig. 1). Experimental evidence supporting such a conception is contained in work of Bodine (1925), Parker (1930), Slifer (1932), and others. Parker (1930), has shown that eggs of *Melanoplus mexicanus*, placed and kept at different constant temperatures from the day laid, hatch most quickly at 27° C. and that on either side of this temperature rates of development are progressively lowered. At 27° C., the optimum constant temperature for this species, the diapause factors (x), when potent and active, are most readily destroyed or reduced in potency to below the threshold value, so that developmental factors (a) again function and normal development follows (Fig. 1). The sporadic hatching of eggs from the same pod under identical temperature conditions is, perhaps, influenced to a great degree by differences in strains of eggs produced, since much evidence, in addition to that given by Parker (1930), has accumulated in this laboratory to justify such a conclusion.

The behavior of diapause-type eggs when kept at temperatures below developmental zeros, 10°–0° C., is in marked contrast to that at constant high temperatures, and studies of these reactions have added much to our knowledge of the workings of both diapause factors (x) and developmental factors (a). In freshly laid eggs (diapause type), put and kept at low temperatures (10°–0° C.) for appropriate periods of time, the diapause factors (x) are completely inhibited or destroyed, since, when the eggs are returned to higher constant temperatures (above developmental zero), normal development ensues, culminating in hatching (Fig. 2) (Bodine, 1925, 1929; Parker, 1930, etc.). Apparently diapause factors (x) present in the egg at laying, are either completely destroyed or their culminative

action inhibited, since they never reach the threshold value as evidenced by the normal development of the egg when subsequently put at higher developmental temperatures (Fig. 2). Low temperatures, below developmental zero (10°–0° C.), naturally cause an inhibition or cessation of developmental factors (*a*), as can be shown by allowing freshly laid eggs to remain at 0° C. for varying periods of time (Figs. 2 and 3; Bodine, 1925, 1929; Parker, 1930). No marked changes in rates of development at higher constant temperatures are noted in eggs thus treated. Possible effects of alternating temperatures exist, but this point will be discussed at some future date.

Not only can destruction or inhibition of diapause factors (*x*) be produced by subjecting freshly laid eggs to low temperatures (10°–0° C.), but eggs in other stages of development (pre-diapause, diapause, or post-diapause) can be shown to be similarly affected (Figs. 2, 3, and 4). Marked differences in degree of effects, however, are noted which are quantitative rather than qualitative. Eggs kept at constant temperatures (above developmental zero) from time of laying to diapause, as shown in Figure 1, have progressively greater or more potent amounts of diapause factors (*x*) and require more extensive treatments with low temperatures to bring about a destruction or inhibition of these factors (*x*) (Figs. 2, 3, and 4). After beginning of diapause, when the potency or concentration of diapause factors (*x*) normally decreases, low temperatures (10°–0° C.) produce a very marked reaction in the further and more rapid destruction of them (Fig. 4). Evidence illustrating this point is obtained by noting hatching time as well as by following rates of oxygen consumption for eggs thus treated (Bodine, 1925, 1929; Parker, 1930). Diapause factors (*x*), therefore, seem especially sensitive to low temperatures (10°–0° C.) and can be completely destroyed or inhibited in their action, while developmental factors (*a*) are depressed or put into a state of cessation of activity from which they quickly recover when temperature is raised above developmental zero.

SUMMARY AND CONCLUSION

1. In certain grasshoppers two types of eggs are produced—a non-diapause and a diapause type.

2. Diapause eggs contain factors (*x*) which cause diapause in

rather definite fashion and at definite stages in the egg's development.

3. Evidence is presented for considering action of diapause factors (x) as of an "all or none type" of reaction.

4. Diapause factors (x) are extremely susceptible to low temperatures (below developmental zero) and can be completely destroyed or inhibited by appropriate exposure to these temperatures.

5. A slow gradual destruction or loss of potency of the diapause factors (x) occurs at constant high temperatures above developmental zero. An optimum temperature exists for certain species.

6. For further analyses of diapause problems in such eggs, detailed studies on single eggs from controlled stock seem necessary.

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THE EFFECT OF THYROID HORMONE UPON GILL REDUCTION IN TRITURUS VIRIDESCENS

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WHILE the larval, gill-bearing forms of the Salamandridae[2] (even those which retain the gills in nature) react upon administration of thyroid gland with a speedy reduction of the gills, the Proteidae, *Siren* and probably *Pseudobranchus*, fail to reduce the gills when injected with thyroid substance. In a recent communication (Morgan and Sondheim, 1931) it was reported that inhibited gill-bearing larvae of *Triturus viridescens* collected in Woods Hole did not reduce their gills by implants of thyroid gland. If this observation is correct, this form would be an exceptional case among the Salamandridae regarding its reaction upon thyroid substance; it would have to be grouped, in this respect, with the Proteidae, *Siren* and *Pseudobranchus*.

In the present article there will be reported experiments to test the responsiveness of gill-bearing *Triturus viridescens*, from the region of Woods Hole, to administration of thyroid substance. The exceptional behavior of these newts, reported by Morgan and Sondheim, could not be confirmed. These animals, when injected with thyroid extract, reduced their gills rapidly, as do other forms of salamanders used commonly in such experiments. It was found, however, that the temperature plays an important rôle in the result: at low temperatures a dose of thyroid substance which at higher temperature produces rapid resorption of the gills is ineffective—a relation which was to be expected from the work of previous investigators.

[1] This work was supported by the Wheeler Research Fund of the University of Maryland Medical School.

The author takes pleasure in expressing to Dr. Eduard Uhlenhuth, his appreciation of the continued interest and criticism which he received throughout the course of his work.

[2] Nomenclature used here is the one adopted in Steineger and Barbour, *A Check List of North American Amphibians and Reptiles* (Cambridge, 1917).

I. TYPES OF WOODS HOLE NEWTS WITH RELATION TO METAMORPHOSIS

In the vicinity of Woods Hole, neotenous *Triturus* occur (Noble, 1929). A large part of these populations exhibit a delayed or inhibited metamorphosis, the most characteristic feature of which is the retention of the basal part of the gills as small stubs which generally lack the filaments. Other larval structures which may be present in such forms include the gill slits, gular fold, larval facial angle, and the dorsal body fin, all of which may show, however, more or less transition to the adult condition. In such animals the gill stubs may not be lost until the animal is of adult size and sexually mature. It

TABLE I

RELATION OF GILL LENGTH TO TOTAL LENGTH IN 250 ANIMALS FROM WHITMORE'S AND MASHPEE PONDS, AND THE PERCENTAGE OF ANIMALS OF ADULT SIZE HAVING GILLS AND GILL SLITS, AT INTERVALS OF 10 MILLIMETERS

Total Length (Mm.)	Average Gill Length (Mm.)	Percentage with Gill Stubs	Percentage with Gill Slits
41–50	0.82	98	98
51–60	1.17	(no data)	(no data)
61–70	0.74	74	20
71–80	0.67	77	15
81–90	0.37	62	16
91–100	0.24	37	15

might be thought, therefore, that such forms are neotenous. However, as will be seen from an inspection of Table I, the lengths of the gills in animals of this type measuring from 40 to 100 mm. total length show a gradual decrease with increasing total length of the animals.

The gill stubs and the gill slits also show a decreasing frequency (measured in percentage in the table) as the animals get larger. The hyobranchial apparatus, according to Noble (1929), also shows various transitional conditions from the larval form to the adult form. Animals of this type are, therefore, not in a stable larval condition, but rather in a gradual process of transformation, which may be completed only at adult size; and this characteristically slow transformation excludes such inhibited forms, even though sex mature, from the true neotenous types which show all the larval character-

istics and are sex mature. True neotenous forms are, in relation to the foregoing types, of slow metamorphic forms, very infrequent; and in our collections in 1930 and 1931, from four different ponds of the Cape Cod region, only 1 per cent of 900 animals of the adult size range were neotenous on this basis.

II. TYPE OF ANIMALS USED IN THE PRESENT EXPERIMENT

From the following description of the animals used in this series, it will be seen that these animals are of the inhibited type referred to above, showing all the characteristics mentioned, and retaining the gill stubs over a considerable period (from January 25 to April 14) in the case of the uninjected control animals. As far as can be surmised from the brief description given by Miss Morgan and Sondheim of their material, the animals used in their experiments are of the same type as those employed in the present series. Fifty-three *Triturus viridescens*, collected at Whitmore's Pond, were received from Woods Hole, January 25, 1932, and were used in these experiments. The total length ranged from 39 to 59 mm., with an average of 51.9 mm. Gill stubs of varying length were present in 98 per cent, and gill filaments were present on 77 per cent of the gills. Gill slits were present in 98 per cent but were very much reduced. The dorsal body fin was present in only 11 per cent, and in these was reduced. The gill stubs ranged in length from 0.45 to 3.15 mm., with an average length of 1.24 mm.—average of the right and left third gill. The gular fold was present in only 5 per cent, and the facial angle was of the adult type in the majority (93 per cent). Complete shedding of the skin was observed in all shortly after the animals were received.

From the foregoing it will be seen that these animals have undergone many of the metamorphic processes but that these processes have not progressed in equal degrees—an independence in the metamorphosis of different organ systems first demonstrated experimentally by Uhlenhuth (1919). The gills and associated structures seem to be the most persistent larval structures; and hence, from a measurement of their size, an indication of the final completion of transformation may be arrived at.

III. EXPERIMENTS AT HIGH TEMPERATURE

Two series of animals were injected with thyroid (Fenger)[3]—one series at 12°–17° C., and the other at 6°–10°. The thyroid was injected in Ringer's solution, its concentration being 100 milligrams per cubic centimeter, each animal receiving 1 minim (equal to $_1{}^1{}_6$ cc.) at each injection. The right and left third gill were measured by means of an ocular micrometer, under a binocular microscope, and the average taken as the gill length of the animal; and the mean gill length of the group was determined.

At 12°–17° six animals received a total of ten injections, there being eleven uninjected control animals. All the experimental animals after the tenth injection showed complete reduction of the gills, the controls showing a reduction of 52 per cent during the same time interval. After the eighth injection the experimental animals showed a reduction of the gills of 79 per cent, the uninjected control animals showing a reduction of 30 per cent at this time.

From the aforementioned data it is concluded that thyroid, injected in the concentration indicated, effects complete resorption of the gill stubs in animals of the inhibited metamorphic type ranging in size from 39 to 59 mm., when injected at 12°–17° C. Injected animals at this temperature showed an acceleration over uninjected animals amounting to 49 per cent.

IV. EXPERIMENTS AT LOW TEMPERATURE

At 6°–10° no significant acceleration of injected animals over uninjected animals was found, when the same concentration and frequency of injection were used which produced an acceleration of 49 per cent at the higher temperature. The lower temperature therefore inhibited the action of thyroid substance, the inhibition amounting to at least 47 per cent. Low temperature in itself exerts an inhibiting effect upon gill reduction; at 6°–10° C. this inhibition amounted to 10 per cent. These results are summarized in Table II.

SUMMARY

The foregoing results, indicating that animals of the particular inhibited type described above are able to utilize thyroid to complete

[3] The author is greatly indebted to Dr. Frederick Fenger, under whose direction the thyroid powder was prepared in the laboratories of Armour and Company.

their metamorphosis, which normally is completed very slowly in nature, would exclude these forms from the permanently gilled types which are non-responsive to such substances (Proteidae, *Siren*, *Pseudobranchus*). Moreover, it is obvious that the effectiveness of thyroid is conditioned very decidedly by an environmental factor—the temperature; it is therefore necessary to record the temperature

TABLE II

EFFECT ON GILL REDUCTION OF THYROID INJECTION IN RELATION TO TEMPERATURE, AND OF TEMPERATURE ON NORMAL GILL REDUCTION

	At High Temperature (12°–17° C.)		At Low Temperature (6°–10° C.)		Effect of Temperature	
	Exp.	Control	Exp.	Control	12°–17° C.	6°–10° C.
Number of animals	6	11	10	16	11	35
Number of injections	8	0	8	0	0	0
Days	16	16	16	16	16	16
Average percentage in reduction in gill length	79.6	30.3	18	16	30.3	20
Difference	49.3		2		10	

NOTE: In the experimentals and controls at low temperature, no measurement was made at the time of the eighth injection, a measurement being made at the time of the seventh injection and 2 weeks later, thus giving ample time for any gill reduction to occur. The difference between these two measurements was less than 1 per cent.

when reporting experiments of this nature. It is not implied, however, that a sufficiently high dosage of thyroid at the lower temperature would not effect completion of transformation in these animals.

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RESPIRATORY REGULATION IN THE LEECH (HIRUDO MEDICINALIS)

(One figure)

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INTRODUCTION

WHILE much has been published on the respiratory regulation of various invertebrates, there is little data available on annelids. Thunberg (1905) reported that *Lumbricus* could be induced to increase its oxygen intake as the environmental oxygen tension is increased. The oxygen tension tested ranged from 20 to 97 per cent, and apparently no attempt was made to test the effect of lower tensions. Recently, Dolk and Paauw (1929) showed that the respiratory rate of the earthworm was independent of the oxygen tension over a considerable range. Amberson, Mayerson, and Scott (1924) found that in *Nereis* the oxygen intake was in direct proportion to the amount of oxygen in the water at any given time. More recently, Hyman (1932), working on the same species, was unable to agree entirely with the results of Amberson, Mayerson, and Scott (1924) and concludes that the rate of oxygen consumption is independent of oxygen tension of the surrounding water if the animals are tested in an environment simulating their natural habitat. From the foregoing data it would appear that no uniformity of respiratory regulation exists in this group of animals. For this reason it seemed worth while that a study be made of another form.

Because of the adaptability of the method employed, the leech (*Hirudo medicinalis*) was used. The experiments presented here were undertaken for the purpose of ascertaining to what extent the oxygen consumption of this form depended upon the oxygen tension of the water to which it was subjected.

MATERIAL AND METHODS

The leeches were obtained from the General Biological Supply House in Chicago, and upon arrival were placed in large jars of tap water for at least 2 weeks without food. They are a large variety, extremely hardy, and thrive well under laboratory conditions. By the end of 2 weeks the animals became acclimated, and the food in the digestive tract had been largely eliminated, therefore decreasing variations due to digestive activity.

The method employed for the determination of the oxygen consumption was essentially the same as the methods used by most workers (Helff, 1928; Hyman, 1932), using Birge and Juday's (1911) modification of the Winkler method for measuring dissolved oxygen in water; 1/200 N sodium thiosulphate solution was used for titration. It suffices to say that the respiratory chamber consisted of a large bottle having a capacity of approximately 6 liters. Into the neck was inserted a three-hole stopper, through which extended two inlet tubes and a stirring device. A glass outlet tube for withdrawing samples of water extended into the bottle through a neck provided in the side.

The general procedure followed in all experiments was as follows: the testing bottles were filled with water and placed in a water bath kept constant at $25° \pm 0.01°$ C. until the temperature of the water was the same as that of the bath. Between twenty and twenty-five animals were placed in the bottles, and the stopper was sealed in with vaseline. About $\frac{1}{2}$ hour was allowed for the animals to become acclimated before the test was started. At the end of this period a sample was withdrawn into testing bottles and analyzed for oxygen content. The water withdrawn was immediately replaced with fresh water of approximately the same oxygen content. At various intervals throughout the course of the experiment the same procedure of testing the oxygen content of the water was followed, until the animals had reduced the tension to the desired level.

The water employed in replacing that quantity used for analysis was prepared by mixing boiled water with tap water until the desired oxygen content was obtained. Preliminary experiments revealed that the addition of several hundred cubic centimeters of water of approximately the same oxygen content failed to alter ap-

preciably the tension of the water in the test bottles. Slight reductions in the volume of the water in which the animals were being tested altered the results considerably.

RESULTS

When the leeches are placed in the respiration apparatus, they usually attach themselves to the sides of the container and remain inactive throughout the course of the experiment. Consequently, the rate of oxygen consumption is a fairly constant index to the metabolic rate. By employing a constant volume of water, an analysis of the oxygen content from hour to hour provides a simple method for graphic treatment of the data. By plotting the amount of sodium thiosulphate required for titrating each successive hourly sample of the water as a function of the time, one obtains a comparative picture of the rate of oxygen consumption. Moreover, knowing the volume of the water tested, it is possible to calculate the percentage saturation of oxygen at any instant.

The results reported in this paper are based upon twenty experiments dealing specifically with the relationship of oxygen consumption to oxygen tension. Figure 1 represents the results of three experiments which are typical of all the others. Each point on the graph represents the amount of 1/200 N sodium thiosulphate required to titrate the amount of dissolved oxygen in the sample of water withdrawn from the respiration chamber from time to time while the animals reduced the oxygen content by their own respiration. An inspection of the graph reveals that there exists a linear relationship between oxygen consumption and oxygen tension above 20 per cent saturation. The results here presented indicate that the rate of oxygen consumption of the leech (*Hirudo medicinalis*) is independent of oxygen tension over a considerable range. Determinations for most experiments were made between the range of 10 and 100 per cent saturation of oxygen. With very few exceptions the oxygen intake was constant from hour to hour down to 20 per cent saturation. In a few cases 25 per cent was the lower limit. Below 20 per cent saturation the animals began to show signs of distress and the rate of oxygen intake diminished rapidly, and the experiments were usually concluded when the tension reached 10 per cent satura-

tion. Below 10 per cent the hourly consumption was so small that it was difficult to determine with accuracy.

DISCUSSION

The method employed in these experiments, that of allowing the animals to lower the oxygen tension of the water by their own respiration, has been the subject of controversy for some time. Attempts

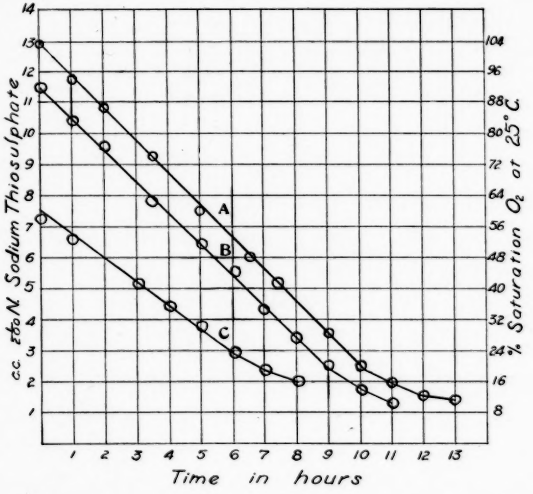

FIG. 1.—The rate of oxygen consumption of the leech (*Hirudo medicinalis*) for decreasing oxygen tensions. Three experiments: (*A*) twenty-four animals, (*B*) twenty-four animals, (*C*) twenty animals. Abscissae, cubic centimeters 1/200 N sodium thiosulphate. Ordinates, time in hours.

have been made to attribute variations in the rate of oxygen consumption to the accumulation of the waste products of metabolism. The lower rate of oxygen consumption at the lower oxygen tensions might well be conditioned by the accumulation of carbon dioxide and the consequent lowering of the pH. In view of this possibility, it was considered advisable to check this point by a series of control experiments. During the course of several tests the water in the experimental bottles was replaced several times with water of the same oxygen tension as that removed. Usually, four changes were

made, the last being at approximately 35 per cent saturation. In no case did the oxygen consumption deviate appreciably under these conditions from that obtained when the same water was used throughout the experiment. Moreover, the lower limit of respiratory regulation was found to be practically the same as that obtained in the other type of experiment. In view of these experiments and the constant results obtained throughout the course of the work, the author believes that the experimental conditions were well controlled and that the method can be satisfactorily employed.

The data in this paper are of interest when considered in connection with the results obtained in other experiments on the oxygen consumption of annelid worms. Thunberg (1905), working with *Lumbricus*, and Amberson, Mayerson, and Scott (1924), on *Nereis*, found a close relationship between the rate of oxygen consumption and the oxygen tension. Dolk and Paauw (1929) found that respiration in the earthworm was constant over a considerable range of oxygen concentration. More recently, Hyman (1932), working with *Nereis*, found that this form, when placed in glass tubes simulating their natural environment, displayed a constant rate of oxygen intake over a moderate range of oxygen tension.

In view of the wide diversity in the habitat of the two animals, the results obtained on leeches compare quite favorably with those obtained by Hyman for *Nereis*. Leeches are subject to various environmental conditions during the course of a single season and consequently a constantly changing oxygen tension. Conversely, the *Nereis*, a marine form, is not subject to such wide variations of habitat, which consideration would lead one to suspect a less perfect adaptation to variable oxygen tensions. An inspection of the results obtained in the two forms seems to bear out this supposition. Hyman found that, if the animals were removed from glass tubes and tested free in the experimental flasks, the oxygen intake varied considerably from that obtained in the tubes, and under these conditions the oxygen intake was more dependent upon the external oxygen content.

The experiments reported in this paper on leeches were conducted with the animals free in the experimental flasks, and in no case did the oxygen intake vary greatly from hour to hour between 20 and

100 per cent saturation. Concordantly with a constant rate of metabolism, it appears that the leech is more able to adapt itself to varying oxygen tensions than the *Nereis*. From the results obtained on the relation of oxygen tension to oxygen consumption, it would appear that we are justified in concluding that the rate of oxygen consumption of the leech is independent of oxygen tension over a considerable range.

SUMMARY AND CONCLUSIONS

1. The relationship of the oxygen consumption to oxygen tension was tested for the leech (*Hirudo medicinalis*). Birge and Juday's modification of the Winkler method was employed for the determination of the dissolved oxygen in the water.

2. A graphic representation of the data reveals the existence of a linear relationship between oxygen consumption and oxygen tension of water between 20 and 100 per cent saturation of oxygen at 25° C. Below 20 per cent saturation the rate of oxygen consumption is less rapid.

3. From the data presented, it is concluded that the oxygen consumption of the leech is independent of oxygen tension between 20 and 100 per cent saturation of oxygen.

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THE AXIAL RESPIRATORY GRADIENT: EXPERIMENTAL AND CRITICAL

(One figure)

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IN THE following paper new data on the respiratory gradient or gradients in *Nereis virens* obtained by the Winkler method are presented. The work was undertaken in order to determine whether reinvestigation of this species by this method would confirm Parker's recent results on CO_2 production (Parker, 1929) or the earlier data of Hyman and Galigher (1921). In view of the recent criticisms by Parker (1929) and Shearer (1930) of the value of the Winkler method, the data on respiratory gradients, and the gradient theory in general, it has been found necessary to devote some space to critical discussion and to pointing out and emphasizing some of the precautions necessary and the difficulties involved in determining respiratory rates in isolated pieces of animals.

EXPERIMENTAL

The work was done at the Marine Biological Laboratory at Woods Hole during the summer of 1930. The species employed was *Nereis virens* Sars. The worms used were freshly dug either by myself in the Eel Pond or by the laboratory collectors. They were used either the same day as dug or the following day. In the former case, the intestine contained ingested material which it was impracticable to remove. Consequently, in many of the experiments, the worms were kept overnight in crystallizing dishes provided with lengths of glass tubing into which the worms crawled. By the following day the intestine had been emptied. No difference, however, was seen in the results whether or not the intestine was empty. The specimens used were large, varying in weight from 2 to 10 gm. each, in length from 3 or 4 to 8 or 10 inches. They were thus very much larger than any individuals employed by Parker (weight about 160 mg.), and the

difference in size is probably an important factor in the difference between Parker's results and mine. Not only is the absolute rate higher, the smaller the animal; but the stimulation of section lasts longer, the smaller the pieces.

For the experiments the head and first few segments containing the more highly differentiated part of the digestive tract were cut off and discarded. The posterior inch or so was also discarded. The remaining part was not cut into three equal pieces, for my experience with annelids has indicated that the low part of the gradient is not in the middle but anterior to the middle. Therefore, three pieces were cut as indicated in Figure 1. The anterior piece was taken directly behind the anterior cut, the middle piece directly behind this. Then a portion of the body was discarded, the posterior piece being cut from the posterior half of the worm. The pieces were 1–2 inches long, depending on the size of the worm, and were of about equal length in any given case, although, of course, of unequal weights, owing to the decrease in diameter posteriorly. The animals were in the asexual state.

After cutting, the pieces were placed in 500 cc. Erlenmeyer flasks with sea water, and the water repeatedly changed until no more blood issued from the pieces. This washing usually occupied about half an hour after cutting. The experiments were then begun. The three flasks containing the respective pieces were all filled by siphon from an elevated receptacle of air-saturated sea water, which was allowed to overflow

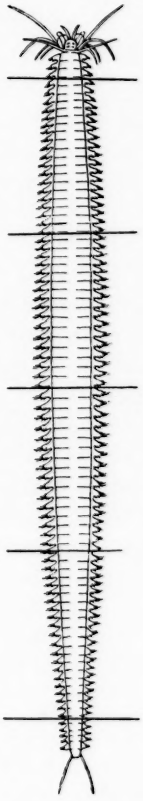

FIG. 1.—Diagrammatic outline of *Nereis virens*, indicating approximate levels of section. The three pieces used in the determinations of oxygen consumption are the first, second, and fourth indicated in the figure, a few segments at anterior and posterior ends being discarded.

from them for some time. The flasks were then closed air-tight, placed on their sides in a large tank of sea water, and covered with a dark cloth. The temperature was that of the running sea water at Woods Hole, which varied from day to day, but very little within the course of one day. Temperatures ranged in different experiments from 21° to 24° C., but all of the results have been calculated for zero. After the lapse of a definite period of time, usually 3 hours, a sample of water was drawn by siphon from each flask and the oxygen content of this and of blank samples determined by Winkler's method. The reliability of this method is considered in the following section. In each experiment one to three worms were used, depending on their size; and corresponding pieces, when two or three were employed, were placed in the same flask. At the end of the experiment the pieces were gently rolled on filter paper until they no longer wet the paper, then placed in a closed weighing tube and weighed.

Preliminary trials soon showed that consistent results could not be obtained within the first 3-hour period after cutting, because of respiratory changes resulting from the cutting itself. It was finally considered necessary to run two or three successive 3-hour trials on the same pieces in order to get some idea of the changes which the pieces undergo. In a few cases two 4-hour periods were employed. This factor of time after section apparently was not sufficiently considered by Parker. He says that pieces were tested "after a lapse of a few hours" after cutting, but gives no definite times; nor is it stated how long a time was occupied by the tests.

The question of the relation of the oxygen consumption of *Nereis* to the oxygen content of the water (a matter also completely disregarded by Parker) has been considered in full elsewhere. It was thought necessary to work out this matter, owing to the claim of Amberson, Mayerson, and Scott (1924) that the oxygen intake of *Nereis* is entirely dependent on oxygen tension. It was found that the dependence is less marked than these workers thought, after the animals have been under the experimental conditions for 2 or 3 hours. The effect is apparently largely or entirely the result of removing the animals from their tubes and keeping them free in water. Within tubes, the oxygen intake is little affected by external oxygen

tension. In the experiments on pieces, the oxygen content of the water was never reduced more than 1 cc. per liter during a 3-hour test, and usually not more than 0.5 cc. per liter. Judging from the results presented in Table II of the paper in question (Hyman, 1932), this decrease in a long-time experiment does not affect the oxygen intake to any appreciable extent. Further, in different experiments the reduction in oxygen content of the water varied among the various pieces without relation to their level, so that this factor would cancel out.

The question of movement was not satisfactorily solved. In pieces free in the water, as in these experiments, movement could not be eliminated. The anterior and middle pieces moved more or less. It is not possible to make any statement as to the relative amount of movement in these two levels. All that can be said with certainty is that the posterior pieces were always very much quieter than anterior and middle pieces, often practically motionless. Since their rate usually exceeded that of the middle pieces, movement cannot be considered the decisive factor in the results. Movement could probably be avoided by putting the pieces in lengths of glass tubing. Unfortunately, this idea did not occur to me until after the experiments had been completed.

Fifteen experiments were performed, of which all the essential data are presented in Tables I and II. The actual oxygen contents of the water have been omitted. In Table I are given all of the data for successive 2- and 3-hour or 4-hour periods. There has been no selection of results; all experiments performed are presented. The table shows the change in oxygen consumption in cubic centimeters per 3 or 4 hours in successive periods after cutting. From the table the following deductions may be made.

1. The oxygen intake of the anterior pieces either does not change with time after cutting or (in most cases) rises with the passage of time. Marked rises are shown in Nos. 25 and 26 and less marked in nos. 27 and 28. Numbers 37 and 38 exhibit a decline with time. In all cases, however, the second and third figures are near enough alike to furnish a reasonable basis for calculation of the rate of oxygen intake. The variation between them does not exceed 20 per cent, and in many cases is less than this amount.

2. The middle pieces exhibit the greatest change, which is to be expected, since all of our work indicates that the lowest levels of a gradient are the most stimulated by cutting. In most cases the rate

TABLE I

OXYGEN CONSUMED, CUBIC CENTIMETERS PER 3 HOURS, BY ANTERIOR, MIDDLE, AND POSTERIOR PIECES OF *Nereis virens*, DURING THE FIRST, SECOND, AND THIRD 3-HOUR PERIODS AFTER SECTION, SHOWING CHANGES WITH TIME AND WITH REFERENCE TO LEVEL

Other details in text.

No. of Exp.	Time	Oxygen Consumed (Cc. per 3 Hours)			No. of Exp.	Time	Oxygen Consumed (Cc. per 3 Hours)		
		Ant.	Mid.	Post.			Ant.	Mid.	Post.
15.....	1st 3 hr.	0.23	0.20	0.18	28.....	1st 3 hr.	0.30	0.27	0.30
	2d 3 hr.	0.22	0.13	Lost		2d 3 hr.	0.26	0.23	0.18
						3d 3 hr.	0.34	0.24	0.20
17.....	1st 3 hr.	0.22	0.28	0.24					
	2d 3 hr.	0.21	0.16	0.15	29.....	1st 3 hr.	0.35	0.39	0.38
						2d 3 hr.	0.34	0.29	0.24
18.....	1st 3 hr.	0.10	0.35	0.12		3d 3 hr.	0.44	0.31	0.32
	2d 3 hr.	0.12	0.18	0.11					
					33.....	1st 3 hr.	Not tested		
19.....	1st 3 hr.	0.19	0.24	0.31		2d 3 hr.	0.43	0.29	0.24
	2d 3 hr.	0.27	0.17	0.23		3d 3 hr.	0.42	0.25	0.26
	3d 3 hr.	0.32	0.20	0.25					
					37.....	1st 3 hr.	0.46	0.41	0.45
20.....	1st 3 hr.	0.24	0.24	0.42		2d 3 hr.	0.42	0.37	0.36
	2d 3 hr.	0.25	0.18	0.32		3d 3 hr.	0.35	0.22	0.21
25.....	1st 3 hr.	0.27	0.39	0.37	38.....	1st 3 hr.	0.57	0.48	0.64
	2d 3 hr.	0.49	0.29	0.38		2d 3 hr.	0.42	0.35	0.40
	3d 3 hr.	0.55	0.35	0.39		3d 3 hr.	0.41	0.36	0.26
26.....	1st 3 hr.	0.28	0.15	0.14	39.....	1st 4 hr.	0.60	0.38	0.35
	2d 3 hr.	0.37	0.18	0.20		2d 4 hr.	0.51	0.27	0.26
	3d 3 hr.	0.43	0.24	0.17					
					40.....	1st 4 hr.	0.38	0.47	0.24
27.....	1st 3 hr.	0.34	0.38	0.41		2d 4 hr.	0.37	0.33	0.17
	2d 3 hr.	0.38	0.36	0.27					
	3d 3 hr.	0.40	0.40	0.31					

of the middle pieces is about as high as, or even higher than, that of anterior and posterior pieces during the first 3-hour period, and declines with time, often markedly so. Here, again, the second and third figures agree reasonably in most cases. Number 37 is the chief exception, the third figure being considerably lower than the second. In some experiments the middle pieces show a rise in the third period as compared with the second.

3. The posterior pieces also decline in rate with time in most of the experiments; Nos. 37, 38, 20, and 27 are among those showing considerable change.

From these data it becomes difficult to decide just when a "basal rate" for cut pieces of *Nereis* can be obtained. I see no reason to doubt that the changes recorded in Table I are actual changes within the pieces themselves. It must be borne in mind that we are dealing with pieces that cannot regenerate heads and which are slowly deteriorating in physiological condition and dying. This change is most rapid in the posterior pieces, and this probably accounts for the decrease in their respiratory rate with time. The changes in the middle pieces are more probably associated with effects of section.

If the figures from the first 3-hour period are used to calculate the respiratory rate, only irregular results are obtained. But if the figures for the second period, when only two periods were run, or the average of the second and third periods, are taken as the basis of calculation, then a definite result appears. Furthermore, the data for the second and third periods agree with each other much better in most experiments than data for the first and second periods. Consequently, the final calculations are based on the oxygen consumed for the second period or for the average of the second and third periods, when three periods were run.

These final calculations are presented in Table II, where also the weights, the size, the number of pieces per flask, and the time since collecting the worms are given. The rate of respiration, as oxygen consumed per gram per 3 hours, gives the basis for comparison between pieces of different levels. It is not assumed that this rate represents the actual rate of such pieces when they are part of the intact worm. It is probably impossible to obtain the real basal rate in forms like *Nereis* where pieces cannot regenerate (heads) and gradually die. It is believed, however, that the figures represent the best that can be achieved under the circumstances.

As shown in Table II, of fifteen experiments, the anterior pieces, under the circumstances stated, consume oxygen at a higher rate per unit weight per unit time than the middle pieces, in fourteen cases. In one case, No. 18, the rates of all three pieces are equal; but since all of the rates are very low in this experiment, it seems probable

TABLE II

FINAL DATA ON PIECES OF *Nereis virens*, SHOWING WEIGHT AND OXYGEN CONSUMED PER GRAM PER 3 HOURS FOR ANTERIOR, MIDDLE, AND POSTERIOR PIECES

Data on oxygen consumed are for the second period or the average of the second and third periods given in Table I. Oxygen calculated for zero and 760 mm. pressure.

No. of Exp.	No. and Size of Worms	Time since Collection	Level	Oxygen Consumed (Cc. per 3 Hours)	Weight in Grams	Oxygen Consumed (Cc. per Gram per 3 Hours)
15	2 Medium	Same day	Ant.	0.22	0.6882	0.32
			Mid.	0.13	0.5844	0.22
			Post.	0.18	0.3855	0.46
17	1 Large	Same day	Ant.	0.21	0.8788	0.24
			Mid.	0.16	0.9130	0.17
			Post.	0.15	0.6366	0.23
18	2 Medium	Same day	Ant.	0.12	0.8700	0.14
			Mid.	0.18	1.2722	0.14
			Post.	0.11	0.7630	0.14
19	2 Medium	Same day	Ant.	0.295	1.2115	0.24
			Mid.	0.185	1.1066	0.16
			Post.	0.24	1.2232	0.19
20	2 Medium	Same day	Ant.	0.25	0.8182	0.30
			Mid.	0.18	0.8160	0.22
			Post.	0.32	0.7908	0.40
25	2 Medium	Next day	Ant.	0.52	1.4137	0.36
			Mid.	0.32	1.4600	0.22
			Post.	0.385	1.0312	0.37
26	2 Smaller	Next day	Ant.	0.40	0.8069	0.49
			Mid.	0.21	0.7482	0.28
			Post.	0.185	0.5362	0.34
27	2 Medium	Next day	Ant.	0.39	1.1184	0.35
			Mid.	0.38	1.3962	0.27
			Post.	0.29	0.9418	0.31
28	2 Smaller	Next day	Ant.	0.30	0.8716	0.34
			Mid.	0.235	0.9612	0.24
			Post.	0.19	0.6712	0.28
29	2 Medium	Next day	Ant.	0.39	1.2210	0.32
			Mid.	0.30	1.3274	0.22
			Post.	0.28	0.774	0.36
33	1 Large	Next day	Ant.	0.425	1.4148	0.30
			Mid.	0.27	1.457	0.18
			Post.	0.25	1.182	0.21

TABLE II—*Continued*

No. of Exp.	No. and Size of Worms	Time since Collection	Level	Oxygen Consumed (Cc. per 3 Hours)	Weight in Grams	Oxygen Consumed (Cc. per Gram per 3 Hours)
37	1 Large	Same day	Ant.	0.385	1.2072	0.32
			Mid.	0.295	1.209	0.24
			Post.	0.285	0.8882	0.32
38	2 Medium	Same day	Ant.	0.415	1.3426	0.39
			Mid.	0.355	1.5412	0.23
			Post.	0.33	1.419	0.23
39	3 Very small	Same day	Ant.	0.39	0.7082	0.55
			Mid.	0.20	0.6398	0.31
			Post.	0.19	0.4898	0.39
40	3 Very small	Same day	Ant.	0.27	0.524	0.51
			Mid.	0.24	0.6014	0.39
			Post.	0.12	0.3758	0.32

that some unknown factor has affected the physiological condition. In twelve of the fifteen cases, the posterior pieces have a higher rate than the middle pieces. The rates of the posterior pieces are probably all too low, as compared with those of the other pieces, owing to the more rapid deterioration in physiological condition of this region of the worm. This change almost certainly accounts for the low rate of the posterior pieces in Nos. 38 and 40. In the intact worm the rate of this region would undoubtedly be much higher than shown in any of the experiments. When the results are calculated from the data obtained after the first 3 hours, the middle pieces do not show a higher rate of oxygen intake than anterior and posterior pieces in any of the experiments as they do in Parker's data.

A closer examination of the rates of the anterior and middle pieces reveals the interesting point that the rate of the anterior piece is higher, and the difference between it and the middle piece is greater, the smaller the specimens used. The smallest specimens employed were those in experiments No. 39 and 40, where the rate of the anterior piece is notably high. The largest worms, Nos. 17, 33, and 37, in general yield lower rates for the anterior and middle pieces. The conditions determining the very low rates of experiment No. 18 are unknown. The average rate of oxygen intake is 0.34 cc. per gram

per 3 hours for the anterior pieces, 0.23 for the middle pieces; that is, the rate of the anterior pieces is 48 per cent higher than that of the middle pieces. I do not consider the differences between middle and posterior pieces very reliable as regards amount, because obviously the rates of the posterior pieces are all too low on account of rapid deterioration in condition.

The close agreement for worms of similar size, and the differences correlated with size indicate that these data have real physiological significance. It is difficult to see how these conditions could appear if the criticisms of Parker and Shearer are correct. And if the relation between size and respiratory rate has physiological significance, the conclusion is justifiable that the respiratory differences of different body-levels are also significant.

The data indicate the existence in *Nereis* of a U-shaped gradient in respiratory rate, high anteriorly and posteriorly, lower in the middle, and confirm the earlier data of Galigher (Hyman and Galigher, 1921). Parker's results are directly opposed to these; but since he did not make determinations at different periods after section, it is impossible to determine what factors are responsible for his results. It seems probable, however, that the after-effects of section are chiefly concerned in determining the high rate of the middle pieces in his data. We have repeatedly shown that cutting obliterates, or even reverses, the gradient temporarily, because the pieces from lower levels are more stimulated by section than pieces from higher levels. Parker states that he used small worms, though how small he does not say; but in general the stimulation following section increases with decrease in size of the piece. He does not state how long after section his determinations were made; and though he mentions repeated determinations on the same pieces, he gives only averages. But that he was dealing with the stimulation following section, at least in some cases, seems to be clearly indicated by his comparison of anterior pieces with and without a head. The headless pieces, even though they have lost blood and are no longer in connection with the cephalic ganglion, respire at a higher rate than similar pieces with heads. Parker offers no explanation of this rather interesting result. We have found, however, that the head regions of planarians and oligochetes and the apical regions of hydroids are

but little affected metabolically by severing them from the rest of the body. The postcephalic parts, on the other hand, are markedly affected by severing them from the head or apical region, or from regions anterior to them. In short, it is the separation of subordinate from dominant parts which is the primary cause of the stimulation following section. Apparently, in forms with a well-developed nervous system the immediate cause of the stimulation is chiefly the section of the nerve paths. In general, such stimulation is greatest in the parts which in the intact animal were most dependent on more anterior levels, that is, the lowest levels of the gradient. The removal of a head from a piece brings about a temporary rise in the respiratory metabolism of the piece, while the head itself is only slightly, or not at all, affected by being separated from regions posterior to it. It seems probable that Parker made his determinations on *Nereis* pieces too soon after section and therefore determined the effects of section rather than the basal rate, or as near the basal rate as can be obtained in pieces; consequently, he obtained a reversed picture of the gradient.

Other complicating factors which may possibly have been concerned in Parker's results may be briefly noted. It is possible that the middle pieces may have been more stimulated than the others by shaking and by removal from water to air for a considerable period; and there is also the possibility that in the small (presumably young) animals the differential susceptibility of the different regions may be concerned, at least in some cases. Parker found that anterior and posterior regions of the body are more susceptible, his observations on this point being in agreement with earlier work (Hyman, 1916*a*). However, he attributes these differences to the fact that the KCN used as agent enters more readily through the mouth and anus, entirely ignoring the fact that it has been repeatedly shown that entrance of the agent through openings of the body does not account for differential susceptibility. But since the anterior and posterior body regions are more susceptible, it may be that their respiratory rate is more affected by the experimental conditions and decreases sooner than that of the middle pieces. The fact that in Parker's graph (Parker, 1929, Fig. 1) the rates of anterior and posterior pieces are far below, and the rate of middle pieces is only slightly

above the rate of the whole animal, certainly suggests that the anterior and posterior pieces have decreased in rate more than the middle. It may be that the graph represents both the greater stimulation following section in the middle regions and the more rapid decrease in rate in anterior and posterior pieces. In the absence of definite statements, and since Parker has given no information as regards length of time between section and the determinations or the actual figures of successive determinations, it is impossible to interpret his data except to say that, as they stand, they possess no significance in relation to the gradient theory and merely show the need of further, more careful investigation.

CRITICAL

1. *Winkler's method.*—Both Shearer and Parker center their criticism on the fact that my results were obtained by means of the Winkler method of determining dissolved oxygen in water. Parker states that this method "was claimed by Shearer (1924, p. 146) to be unreliable, a conclusion with which I agree." As Shearer, in the paper cited, did not state any reason for his objection, and Parker gives no reason for his agreement, the grounds of objection remain uncertain. Later (1930, p. 264) Shearer made a statement from which one infers that he considers that the presence of organic materials in the analyzed water interferes with the titration. This criticism had already been considered and answered in my first work with the Winkler method (Hyman, 1916*b*, p. 242). As Shearer apparently bases his whole objection on this one point, new checks on this question are presented in Table III. The check consists in adding I-KI solution to water which has, and water which has not, contained animals, and titrating back with thiosulphate to determine the comparative iodine loss. Owing to the volatility of iodine, it is essential that the iodine be added from a burette quickly to both samples. The water must be acidified with HCl before the iodine is added. In making these tests, a given sample of water is divided into two parts, and worms are placed in one. After several hours, a 100 cc. sample is taken from each container, several drops of concentrated HCl added to each, enough I-KI solution run into each from a burette to give a color like that obtained in the Winkler

analysis, and both samples titrated with N/100 sodium thiosulphate to the usual end-point with starch. In some cases 100 cc. distilled water was also compared. In these tests the worms remained in the water very much longer than would have been the case in actual experiments. As seen in Table III, there is no essential difference in the amount of thiosulphate required to neutralize equal amounts of I-KI solution added to fresh, salt, or distilled water, whether or

TABLE III

AMOUNT OF N/100 SODIUM THIOSULPHATE REQUIRED TO NEUTRALIZE EQUAL AMOUNTS OF IODINE ADDED TO EQUAL AMOUNTS OF WATER (100 CC.) IN WHICH WORMS HAVE REMAINED FOR SEVERAL HOURS, WATER FROM THE SAME SAMPLE IN WHICH WORMS HAD NOT BEEN KEPT, AND DISTILLED WATER

The water in each case is first acidified with HCl before adding the iodine. Worms kept in water 6 hours.

PLANARIA				NEREIS			
	Cc. Thiosulphate Required				Cc. Thiosulphate Required		
No.	Water Blank	Water from Worms	Distilled Water	No.	Sea Water Blank	Sea Water from Worms	Distilled Water
1	12.92	12.99	.....	1	12.84	12.83	.....
2	12.83	12.87	.....	2	6.10	6.08	.....
3	12.86	12.82	.....	3	7.95	7.97	.....
4	11.29	11.33	11.29	4	8.01	7.95	.....
5	11.05	10.97	11.01	5	14.35	14.29	.....
6	11.11	11.10	11.11	6	14.68	14.71	14.71
7	13.16	13.17	13.10				
8	12.40	12.46	12.50				
9	11.82	11.76	11.81				

not planarians or *Nereis* had remained several hours in the water. These data should finally dispose of this objection to the Winkler method, at least as regards these species. It may also be pointed out that Shearer's criticism cannot possibly be correct, for if it were, larger masses of animals in the same amount of water would appear to consume more oxygen per unit weight than smaller masses, for they would give off more organic materials and "mucus" into the water. But this is never the case, for the oxygen consumed is always directly proportional within the limits of variation to mass of worms in comparable physiological condition.

Both Shearer and Parker regard my figures of oxygen consumed

per unit weight per unit time as too low; whereas, if the objection of Shearer were correct, my figures would be too high, because the alleged absorption of iodine by the organic materials would appear as oxygen consumed. It would also be remarkable if in every case the amount of "mucus" given off by animals in my experiments were always of just the right quantity to yield a result in accordance with the gradient and other hypotheses. While it is certainly true that planarians and annelids give off much mucus and other secretions, it seems impossible that forms like sponges and *Tubularia* give off enough mucus to account for the axial respiratory differences which have been found in them (Hyman, 1925, 1926).

Shearer states that the Winkler method was "originally introduced as a method of estimating the oxygen content of fluids in pure chemistry." In Winkler's original paper (1888, p. 2851) Winkler says: "Die titrimetrische Methode lässt sich auch zur Bestimmung des in natürlichen Wässern enthaltene Sauerstoffs gut anwenden." Birge and Juday (1911), in their classic work on the dissolved gases in Wisconsin lakes, used the Winkler method for the oxygen determinations after demonstrating its accuracy by comparison with gas analysis methods. The method is also standard in water analysis. As natural waters may contain large numbers of organisms and their secretions, the conditions of analysis are not fundamentally different from those in experiments in which water samples in which animals have lived are analyzed. If necessary, the permanganate correction given in manuals of standard water analysis can be applied. In the light of all the facts, Shearer's position seems to be untenable.

Shearer states that the Winkler method was abandoned by experimentalists for the more accurate manometer method. As a matter of fact, the method is still in use by a considerable number of workers who are using large organisms. The method is obviously not satisfactory in all respects for small organisms or small quantities of material, owing to the fact that large samples are required for the analysis; and it was abandoned by certain experimenters because they wished to work with small quantities of material. The Winkler method is so much more simple than any other known method for determining dissolved oxygen, is so direct and uncomplicated, and so suitable for retaining the animals during the experiment in an

abundance of water and in quiet, undisturbed conditions, that it appears on the whole to be the best method for studying the oxygen intake of aquatic animals of a certain range of size. I know of no case where results obtained by the Winkler method have been actually shown to be erroneous. I see no reason to doubt the accuracy of any of my data obtained by this method. These data conform to a large number of recognized physiological generalizations, such as the effects of age (size), food ingestion, injury, etc., on respiration. It seems impossible to suppose that the method can yield correct results in a large number of cases and be incorrect on this one matter of axial respiratory differences. Until the critics of the method shall have proved its inaccuracy by direct comparison of the oxygen intake of the same animals by the Winkler and by other methods, criticisms such as those of Shearer appear to be without adequate basis.

2. *Shearer's and Parker's methods.*—Shearer places the utmost confidence in the manometer method; yet his readings are highly variable. I have had some contact with workers using manometers and observed that they had great difficulty in obtaining uniform successive readings with material that was capable of locomotion. The only published successive readings on planarians that I know of are those of Krajnik (1922), and there is 60 per cent variation in his figures. Shearer has published nothing but averages. The chief trouble with the manometer method as applied to motile animals is that the constant shaking of the instrument stimulates the animals to muscular activity. The most careful work on planarians by the manometer method is that of Fraps (1930), of which only a small part has so far been published. After long trial of this material, Fraps found that the shape of the chambers is important for accurate work (Barcroft-type chambers being quite unsuitable), that the worms must be kept entirely under water, that the stopping and starting of the instrument (which he now eliminates) to take readings introduces errors, and that, in short, only the most painstaking attention to every detail will yield results of any value with planarian material. The experience of Fraps clearly indicates that the manometer cannot be employed with planarians in the casual manner in which Shearer seems to have worked and that uniform succes-

sive readings can be obtained only after long study and elimination of various sources of error.

With the Winkler method, where the animals are placed in advance in the containers and left until they become quiet, where the containers remain motionless and darkened during the tests, where direct observation at intervals shows the planarians entirely motionless, and where the animals are completely submersed, it is quite simple, with a little practice, to obtain figures in successive trials, which vary less than 10 per cent. I have repeatedly published successive determinations of the same lots of material; thus in an earlier paper (Hyman, 1923, p. 53), I gave two figures, taken at a 24-hour interval, of a number of lots of pieces, showing very little variation. Such slight variation constitutes evidence for the reliability of the method and the correctness of the results. Since only averages are given by Shearer and Parker, the significance of their data remains uncertain. In Parker's method carbon dioxide output is measured, and this has always been considered by physiologists a less reliable measure of respiratory metabolism than oxygen consumption. However, the chief objection to Parker's procedure is that the material is exposed to air.

3. *Movement.*—Basal metabolic rate must be determined on quiescent animals. Shearer admits great variation in his readings because of restlessness of the pieces, but nevertheless uses his data as a basis for criticism of mine. Most sorts of animals can be kept quiet by taking sufficient trouble with the conditions of the experiments. Obviously, a constantly shaking apparatus is not an ideal arrangement for insuring immobility. Some species of planarians are more restless than others; *Planaria dorotocephala* can readily be kept quiet by the procedures already mentioned.

Shearer states that the worms and pieces he used were constantly moving and quivering during the experiments, and believes that different degrees of activity with respect to level accounted for the graded oxygen intake he observed. These things may have been true for the species used, but they are certainly not true for *Planaria dorotocephala*. To test Shearer's contention, I recently observed the behavior of freshly cut halves of the first zooid of *P. dorotocephala*. They moved about for a short time after cutting, the anterior halves

being the more active; but within half an hour all pieces had come to rest and examination with a hand lens magnifying 18 times failed to show the slightest "quivering," such as reported by Shearer. This quivering in his experiments probably resulted from the shaking of the manometer. After the first few hours following section, shorter cut pieces of *P. dorotocephala* often remain motionless during several days, until the new heads develop; and the mucus secreted during this time forms an envelope attached to the wall or bottom of the container and constitutes essentially a thin-walled cyst. It is quite certain that axial respiratory differences obtained in *P. dorotocephala* did not result from axial differences in motility. Furthermore, Shearer's argument cannot be applied to axial respiratory differences observed in *Grantia* (Hyman, 1925) and *Tubularia* (Hyman, 1926), material entirely incapable of movement. Shearer has apparently overlooked the fact that some of the work on respiratory gradients was done with non-motile material. In *Corymorpha* also, the amount of motility is slight. Obviously, Shearer's argument cannot dispose of all of these results while the admittedly great motility in his experiments renders his data valueless as evidence either for or against the gradient hypothesis. However, an axial gradation in motility and irritability such as he found in these species obviously also requires explanation. It must mean the existence of a physiological gradient of some sort in some organ or organs.

4. *Specific differences.*—Shearer states that his work was done on "*Planaria nigra*" and "*Planaria lactea.*" Properly speaking, there are no such species; the correct names are *Polycelis nigra* and *Dendrocoelum lacteum.* These two species are not closely related, belonging, in fact, to different families; and, further, they differ considerably from the genus *Euplanaria* (old name *Planaria*) in which *dorotocephala* is now placed. Since different species respire at different rates, Shearer's criticism of my figures for *Planaria dorotocephala*, because they do not agree with what he considers the rate of oxygen intake of his two species, seems not entirely justified. However, as a matter of fact, the agreement is rather close. Shearer's figure of 0.429 cc. of oxygen per gram per hour given in his Table I (species used not specified) is probably not correct for the basal respiration, since he finds whole worms showing a higher rate of oxygen consump-

tion than halves. This is certainly not true for the species with which I have worked. As I showed in 1923, and as others have confirmed, pieces respire at the same or at a higher rate per unit weight than whole worms, depending on their length. The shorter the piece, the higher the rate of respiration for at least a certain length of time after cutting. There is no marked difference in respiration between halves and wholes. The difference which Shearer found is undoubtedly due to the greater motility of the wholes. Consequently, Shearer's figure for the halves is probably more nearly correct than that for the wholes, although even these are stated to have shown considerable motility. His rate for the halves is 0.25 cc. per gram per hour, which is about the same rate as I find for freshly collected 15–18 mm. *P. dorotocephala*. Much depends on size, which is not considered at all by Shearer.

A brief comparison of Shearer's and Krajnik's data is of interest. Both worked on *Polycelis nigra* and *Dendrocoelum lacteum*, and both used manometer methods. But, whereas Shearer seems to imply that both species have the same rate (as he fails to specify from which species the data presented were obtained), Krajnik found that *D. lacteum* uses about $2\frac{1}{2}$ times as much oxygen as *Polycelis nigra*. Krajnik's figures are 2.37 cu. mm. of oxygen per worm per hour for *Dendrocoelum lacteum* and 0.99 for *Polycelis nigra*. Since weight was not given, these figures are a little difficult to compare with Shearer's figure of 429 cu. mm. per gram per hour (for both species?); but on the assumption that a large worm of either species weighs 20 mg., Shearer's rate is over 8 cu. mm. per worm per hour, or nearly 4 times as great as Krajnik's rate for *Dendrocoelum lacteum* and 9 times as great as for *Polycelis nigra*. Even assuming a weight of 10 mg. per worm. Shearer's figure is still considerably larger than Krajnik's. Part of the discrepancy may perhaps arise from the fact that Krajnik's worms had remained a long time in the laboratory, while Shearer's may have been freshly collected (Shearer gives no information on this point). It is impossible to say which of these two experiments is correct; but the discrepancy is present, although both workers used the supposedly accurate manometer method. The point cannot be too strongly emphasized that in work with naked

motile material a large number of factors have to be considered besides the accuracy of the apparatus.

Parker criticizes the results of Hyman and Galigher (1921) on the rate of oxygen intake of *Nereis*, because the figures do not agree with his, but fails to mention the fact that my data on whole *Planaria doroto cephala* agree very well with his on *P. maculata*. Now *P. dorotocephala* and *P. maculata* are really closely related species (both are now put in the genus *Euplanaria*). My experience with *P. maculata* is that its oxygen consumption is slightly less than that of *P. dorotocephala*. A large specimen (15–18 mm.) of *P. dorotocephala* freshly collected (for respiratory rate gradually declines with life in the laboratory) consumes between 0.25 and 0.30 cc. of oxygen per gram per hour. Translating this into Parker's units, on the basis that 0.08 gm. of oxygen equals 55.987 cc. at zero and 760 mm. pressure, we get a rate of 0.0058–0.0070 mg. of oxygen per gram weight per minute, while Parker reports 0.00503 mg. of carbon dioxide per gram per minute for his largest specimens of *P. maculata*. As we do not know the R. Q. for planarians, it is impossible to pursue the comparison further.

Concerning the data on *Nereis* published by Hyman and Galigher in 1921, I can only say that they were all obtained by Galigher. The work was done at Friday Harbor, Washington; and while one of the species used is said to be identical with the *Nereis virens* of Woods Hole, it is not impossible that some geographic or physiologic difference exists between the Atlantic and Pacific Coast forms. Temperatures are much lower at Friday Harbor than at Woods Hole, and finally the worms used were very much larger than those employed by Parker.

In working with *Nereis virens* at Woods Hole in the summer of 1930, I found the rate of oxygen intake to be considerably higher than that recorded in the paper by Hyman and Galigher. Whether this difference is real, I cannot say. My figures ranged from less than 0.10 cc. of oxygen per gram per hour for the largest *Nereis* used, weighing 5–10 gm., to 0.22 for the smallest, weighing about 2 gm. In Parker's units my highest figure translates into about 0.005 mg. of oxygen per gram per minute. Parker found an average of 0.01 mg. of carbon dioxide produced per gram per minute. But the worms

used by Parker were very much smaller than the smallest tested by me, weighing about 160 mg., or one-twelfth the weight of mine, and could reasonably be expected to consume at least twice as much oxygen per unit weight as my smallest worms. It appears, therefore, that there is no serious discrepancy between my figures for the oxygen intake of *Nereis virens* and Parker's figures for the carbon dioxide output. It is also not impossible that Parker's figures are too high, owing to the stimulating effect of removing the worms from water and testing them in air. Such a procedure cannot be finally accepted until it shall be checked by comparison with animals submersed in water. I have shown elsewhere (Hyman, 1932) that in all probability the respiration of *Nereis* is never "normal" when the worms are removed from their tubes; and certainly, until experimental proof is given, it cannot be regarded as normal when the animals are removed from the water.

5. *Nutrition.*—The state of nutrition of an animal is always a troublesome factor in the study of basal metabolism. In higher animals where digestion occurs exclusively in the lumen of the digestive tract, a starvation period of 1 or 2 days is generally considered sufficient. But forms like planarians, with intracellular digestion, require longer for the disposal of a meal. Concerning this matter, Shearer says (Shearer, 1930, p. 264): "The amount of metabolites present in the tissues . . . . cannot be simply dismissed by starving the animals for a week. It is doubtful if proper allowance can ever be made for this in the case of planarians where one species differs so much from another in the distribution of gut diverticula and their capacity for storing reserve materials." Different species of triclad planarians do not, to my knowledge, differ very greatly in the distribution of gut diverticula or the storage of food. In *Planaria dorotocephala* at least, we have exact knowledge on these matters (Hyman, Willier, and Rifenburgh, 1924; Willier, Hyman, and Rifenburgh, 1925). We found that in this species digestion in the gut is wholly intracellular, that 5 days are required for the complete digestion of the food vacuoles by the entoderm, that food is stored in the entoderm chiefly as fat, and that this stored fat does not disappear even in prolonged starvation. It was further shown that the respiratory increase caused by feeding declines rapidly and that in the course of 1–2

weeks after feeding, the respiratory metabolism of *P. dorotocephala* reaches a constant minimum level. Following this period, the respiratory rate begins to rise as a result of starvation. Consequently, about 2 weeks' starvation is believed to be the proper length of time before respiratory experiments should be conducted. One week, the time employed by Parker and Shearer, is probably sufficient.

6. *Amount of axial respiratory difference.*—Both Parker and Shearer believe that the respiratory differences I obtained in pieces of *Planaria* with reference to the axis are too small. According to my experience, the maximum difference that can be expected between anterior and posterior parts of the first zooid of planarians is 15–20 per cent in the most favorable material. In some species I should expect still less difference. In *P. dorotocephala* the average difference was 12 per cent. From my experience with *P. maculata* I should anticipate still less difference in this species; in fact, in some species of planarians the difference is probably too small for our methods. In various other invertebrates, in some of which both motility and complex organ systems with possible difference in direction of gradients are absent, or are present only in the extreme distal region of the body, much greater axial respiratory differences have been observed. For example, in *Corymorpha* (Child and Hyman, 1926) distal pieces were 21 per cent higher in rate of oxygen consumption than proximal pieces; in *Grantia* (Hyman, 1925) the difference was 25 per cent; in *Tubularia* (Hyman, 1926), 39 per cent; and in *Nereis* in the present experiments, 48 per cent. The differences in general are greater, the smaller (younger) the animal in any given species.

Since Parker and Shearer both maintain that the difference which I reported between anterior and posterior parts of the first zooid of *Planaria dorotocephala* is too small to be significant, I have asked Professor Sewall Wright to examine my data from a statistical standpoint. He has kindly done this and reports as follows: From my Table II (Hyman, 1923, p. 53) in which are given two determinations of the same lots of pieces with a 24-hour interval, the probability is 0.96, showing clearly that there is no significant difference between two determinations of the same pieces. For my Table V (p. 57) the data for the oxygen consumption of anterior and posterior halves of the first zooid give a probability of 0.00006, a figure which proves

beyond any doubt that the difference is significant. The mere fact that a difference is small does not render it insignificant. In the same table, the difference between the second half of the first zooid and the piece from the posterior zooids has a probability of 0.0002, also significant statistically. From this statistical analysis there cannot be any doubt that these differences are significant; but, of course, they may arise from some other constant factor than basal respiratory rate. The preceding consideration of the factors suggested by Shearer—namely, movement and mucus secretion—does not afford any evidence that they play a rôle in the data presented. The belief is, therefore, justified that the figures really represent differences in basal respiratory metabolism of these pieces.

7. *Respiratory rate of different tissues.*—Parker's discussion of the relative rates of respiration of various tissues requires some consideration. He speaks of the low respiratory rate of nerves; but the nerves to which he refers are medullated nerve fibers, a large part of the weight of which consists of inert medullary sheath. Does any one know the respiratory rate of the axis cylinders? According to Meyerhof and Schulz (1929), the resting-rate of the non-medullated nerves of *Octopus* is 5 times and of the spider crab *Maia* 10 times that of the frog medullated nerve, and consequently much higher than the rate of any tissues of the frog as given by Fenn (1928). However, the comparison of respiratory rates of tissues from different kinds of animals is probably futile. Tissues even from different species of frogs cannot be validly compared; and factors such as season, size, and sexual maturity must also be taken into account. In many of the papers on the subject, even the species used is not stated. In the following papers nervous and other tissues are compared within the same species. The resting-rate of the sciatic nerve of *Rana temporaria* averages, at 15° C., 23 cu. mm. of oxygen per gram wet weight per hour (Gerard, 1927); while Bass (1923) gives for whole frog brain (Landfrosch, presumably *R. temporaria*) a rate at the same temperature of 200 cu. mm. in air, about 400 cu. mm. in pure oxygen; spinal cord in pure oxygen averaged 350 cu. mm. These rates for frog central nervous system obviously far exceed those for other frog tissues. While Hill and Nabarro (1895) and Abderhalden and Wertheimer (1921) found brain (birds and mammals) to con-

sume less oxygen than liver and muscle, on the other hand Usui (1912) reported a higher rate for central nervous system than for liver in the frog; and Terroine and Roche (1925*a*, 1925*b*) testing several tissues each of rabbit, hen, guinea pig, pigeon, bat, and a small bird of the genus *Vidua*, found within each species the following order of respiratory rate: brain, kidney, liver, muscle. The experiments of Terroine and Roche seem to me the most careful and extensive which have been performed upon this question. Yet it is to be noted that none of these investigators worked with gray matter alone. Apparently, they took entire brain or samples that would include both white and gray matter (their papers are not specific on this point). Obviously, only the gray matter can fairly be compared with such epithelial organs as kidney and liver. According to Loebel (1925), slices of rat cortex respire at 38° C. at a rate of 5,600–11,200 cu. mm. of oxygen per gram *dry weight* per hour in a non-nutrient medium, and 8,500–15,000 cu. mm. in the presence of glucose. For gray matter *in situ* surrounded by its proper nutrient medium, one can assume a still higher rate. A very high metabolic rate for gray matter (animal used not stated) is also indicated by Krontowski's work (1927) on the sugar utilization of explants. A more recent paper by Holmes also indicates a much higher oxygen consumption in gray matter than in white (Holmes, 1930). Altogether, Parker's position as regards the low metabolism of the central nervous system is quite untenable in the face of all of the facts.

But even if the central nervous system or the nerve cell itself has a low metabolism in the adult animal, the fact is of no fundamental significance as regards the gradient theory. All that this theory maintains as regards this point is that the central nervous system is localized in and develops from, the regions which show the most intense metabolism in the early embryo or larva, and it has been pointed out repeatedly (Hyman, 1916*a*, p. 106; Child, 1921, pp. 100–102; etc.) that it is entirely unnecessary to assume that this intense metabolism must persist throughout life. In short, so far as the conception of physiological gradients and their significance is concerned, Parker's argument concerning the metabolism of the central nervous system is obviously entirely beside the point.

Since the rates of respiration of different tissues after differentia-

tion has occurred may differ widely from the primary rates of the embryonic regions from which they arose, it is evident that the development of particular localized organs at a certain stage of the life-history (such, for example, as the sex organs in planarians) may alter pre-existing axial respiratory differences very greatly. *Planaria dorotocephala* does not develop sex organs under the usual conditions; but in *P. maculata*, which Parker used in his experiments, development of sex organs apparently occurs in all individuals above a certain size, at least in many localities. During the development and functional activity of the sex organs there may be very great changes in the axial respiratory differences. It is possible to determine whether or not this is the case only by studying the respiration of both asexual and sexual stages. Even with all precautions, however, data on respiration obtained during sexual development or at sexual maturity are of value only as showing conditions at that period. To possess any significance in relation to the gradient theory, they must be compared with data from earlier sexual stages and asexual or even developmental stages.

Assuming that Parker's data are correct for the material and conditions of the experiment, he does not even state whether the animals are sexually mature, in some earlier stage of sexual development, or asexual. However, in the vicinity of Woods Hole, where Parker's determinations were made, the large individuals of *Planaria maculata* become sexual in the summer; and since his work was done during the summer season, his animals were probably in some stage of sexual development. In sexual individuals two ovaries and a number of testes develop in the anterior half of the body, a much larger number of testes and a very complicated muscular and glandular copulatory and cocoon-forming apparatus in the posterior half. One cannot consider two such halves as comparable for the purpose of determining fundamental axial differences in respiration as little complicated as possible by differentiation. In the investigation of axial respiratory differences in *Planaria*, results obtained from sexual animals alone must always be of uncertain value unless checked by data from non-sexual individuals.

Even asexual planarians, however, are not entirely satisfactory material. According to the evidence at hand, the gradient in the

body wall of *Planaria*, and probably in the parenchyma also, is antero-posterior, while in the pharynx-gut complex the gradient apparently extends both anteriorly and posteriorly from the "high" pharyngeal region. Apparently, this gradient is decreased, but not wholly eliminated, by starvation. The double gut gradient is unquestionably secondary in development and associated with the special differentiation and function of the pharynx and the greater digestive activity of the middle parts of the gut. It is evident that an organism with such a gradient complex is not the most satisfactory material for investigation of the respiratory gradient in isolated pieces, for the respiratory methods give only the total respiration of the pieces. The total respiration is the sum of the respiratory activity of all organs in the piece; and if different gradients extending in different regions do exist in such organisms, it is evident that a very considerable amount of comparative work is necessary before we can draw any conclusions as regards gradients from our data. It is quite possible that with all experimental precautions isolated pieces of *P. maculata* may show no definite axial differences in respiration, even though such differences are present in *P. dorotocephala*. *Planaria maculata* is a broader, shorter species than *P. dorotocephala*, and it is probable that the amount of gut tissue in relation to other tissues in the middle body region is greater than in *P. dorotocephala*. Presence or absence of a respiratory gradient, as determined in isolated pieces of adult animals, may depend on slight differences of this sort; and its presence in one species does not make it certain that we shall find it in other related species. It should be evident that extended and careful investigation of various stages and nutritive conditions and, if possible, checks of some sort by other methods are necessary before we can interpret respiratory data obtained from isolated pieces in such material. The work on *Grantia*, *Tubularia*, and *Corymorpha* is undoubtedly more significant than that on *Planaria*, since the different axial levels in *Grantia* and the levels below the hydranth in *Tubularia* and *Corymorpha* are morphologically very similar. Moreover, in *Grantia* and *Tubularia* motor activity, except diffuse ciliary activity, is absent and in *Corymorpha* slight. *Nereis* also is not entirely unfavorable material when the

anterior and posterior ends are discarded and the parts are kept in as nearly as possible normal environment.

As regards the sea anemone, *Metridium*, for which Parker gives data, assuming that they are correct, it is, of course, of some interest to know that the animal in its adult form shows no higher CO_2 production in the apical than in the basal half; but the fact is of importance to the gradient theory only as further evidence that gradients are often absent in the later stages of development. However, Parker does not give any evidence to show that the rate of the basal piece is not increased by section or that the foot region was not lacerated in removing the animal from its natural habitat. We should expect that respiration in the basal half would be considerably increased by either of those injuries, and there is nothing to indicate that both of them may not have been involved in Parker's material. Without further investigation these data on *Metridium* obviously have no definite meaning.

SUMMARY

1. New data are presented on the rate of oxygen consumption of anterior, middle, and posterior pieces of medium to large individuals of *Nereis virens*, as determined by the Winkler method with the pieces kept undisturbed in water.

2. Data obtained during the first 3 hours after section are irregular. Data for periods later than 3 hours after section gave, in fifteen experiments, a higher rate for the anterior, as compared with the middle, pieces in fourteen cases and a higher rate for the posterior, as compared with middle, pieces in twelve cases. The average of the fifteen experiments was 0.34 cc. per gram per 3 hours for the anterior pieces; 0.23 for the middle; and 0.30 for the posterior pieces. Since the posterior pieces apparently undergo more rapid deterioration in physiological condition than other pieces, the rate obtained for those pieces is believed to be much too low.

3. Owing to the fact that headless pieces of *Nereis* are incapable of regenerating heads and undergo gradual deterioration in physiological condition, the posterior pieces more rapidly than others, the respiratory rate of pieces of the body isolated by section, whatever the method of determination, is probably a very imperfect picture of the conditions in the intact worm.

4. The rate of oxygen consumption is higher and the difference between anterior and middle pieces is greater, the smaller the worm; but among the larger individuals size differences are not of much importance.

5. In no case among the determinations by the Winkler method was the rate of the middle pieces higher than that of the end pieces, as it is according to Parker's data. It is concluded that his data in their present form do not afford a basis for any definite interpretation.

6. In the critical section of the paper the validity of data on respiration obtained by different methods is discussed. Attention is called to some of the precautions necessary in determinations of respiratory rate on isolated pieces, and certain criticisms advanced by other investigators are considered.

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DETERMINATION OF NEW AXES BY LATERAL GRAFTS AND INCISIONS IN CORYMORPHA IN RELATION TO THE GRADIENT AND DOMINANCE

(Thirty figures)

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IN AN earlier paper (Child, 1929*a*) it was shown that even very small pieces of the naked portion of the stem or hydrocaulus of *Corymorpha palma* transplanted into lateral incisions in the stems of other individuals may act as organizers or dominant regions and determine the development of a new hydranth and a longer or shorter stem. In this development the graft itself gives rise only to the distal part of the new hydranth, the hypostome or hypostome plus more or less of the manubrium, the remainder of the hydranth body and the stem arising from the tissues of the host by reorganization under the influence of the graft. In the paper cited above, some evidence was given indicating that the ability of the graft to act as organizer depends to some extent on the level of the body from which it is taken and the level into which it is grafted.

It was thought desirable to investigate further this influence of body level on organizing ability, particularly since it seemed to show a relation to the axial gradient. The month of July, 1930, was spent at the Scripps Institution of Oceanography in experiment along this line, the results of which are presented below. I wish to acknowledge my indebtedness to the director of the Institution, Dr. T. Wayland Vaughan, for again placing at my disposal the facilities of the Institution and so making possible this continuation of earlier work.

MATERIAL AND METHOD

Corymorpha palma has been so extensively employed as experimental material that description is unnecessary here. The procedure followed in making the grafts was essentially that described in an earlier paper (Child, 1929*a*). In all grafts made for comparison of different levels of the stem (see Tables I–III below), pieces 1–2 mm.

in length were isolated from the desired level by transverse planes of section, were cut into quarters ($\frac{1}{4}$ sectors) by planes of section in the longitudinal axis of the stem and approximately at right angles to each other (Fig. 1), and one of these sectors was implanted in a lateral incision at the desired level of the host stem. The grafts were prepared first, then the incision was made at the desired level, care being taken not to make the incision larger than necessary for admission of the graft, that is, not more than one-third the circumference of the stem. A needle was inserted into the incision and moved about in order to crush some of the large entodermal parenchyma cells and form a slight depression in which the graft might lie. If this is not done, the graft is likely to be forced out by contraction of the stem. With the host stem lying on a glass plate or a tissue-culture depression slide with little water, the graft was laid on the incision and gently pushed in without any attempt at orientation. The host stem was then bent by means of needles, so that the incision was on the concave side and closed or almost closed, and was held in this position for 1–2 minutes, this time being sufficient for adhesion of graft and host tissues to such an extent that loss of a graft occurred very rarely. The grafted stems were left undisturbed for 1–2 hours with enough water to prevent drying, and were then placed in running sea water. The basal region of the stem with its tangle of holdfasts binding together a mass of sand was not removed; and when the stems were placed in running water, the weight of the sand served as an anchor. The first examination was made not later than 3 days after grafting, and later examinations as the material required.

FIG. 1.—One-quarter sectors of transverse pieces of *Corymorpha* stem used as grafts.

The grafts were made in lots of ten, each lot representing grafts from a certain level of the donor stem implanted at a certain level of the host stem. It was found convenient to place the ten stems of each lot together in a finger bowl which was kept in running sea water. In a short time—at most a few hours—the stems had attached to the glass by the growth of new holdfasts, and the lot could be removed for examination at any time with very little disturbance of the individuals.

The animals used were the largest obtainable at the time (July). They ranged during the course of the work from 60 to 25 mm. in length, the size decreasing during the period of experiment. The average size of the animals always decreases during the summer, and they may undergo gradual disintegration and disappear from their natural habitat during the late summer, apparently as a result of the rise in water temperature. At least this is true in the shallow bay from which my collections have been made. The animals become stunted and disappear first from the inner parts of the bay, which are farthest from the single inlet and have the highest water temperature; and as summer goes on, the stunting and disappearance progress along the tidal channels of the bay toward the mouth.

Three levels of the stem—distal, middle, and proximal—were selected for grafting. The distal level was 7–15 mm. proximal to the distal hydranth (Fig. 2, *A*), depending on the length of the individual. This distance from the hydranth was chosen because it had been observed that, when the distal hydranth was removed and a new hydranth reconstituted, about that range of stem length was involved in hydranth development, so that in the longer individuals a graft as much as 15 mm. from the distal hydranth might become part of a new hydranth after removal of the original. However, at this distance the graft is far enough proximal so that, if it determines a new axis, a sufficient portion of that axis will develop to involve host tissue as well as graft. The middle level was approxi-

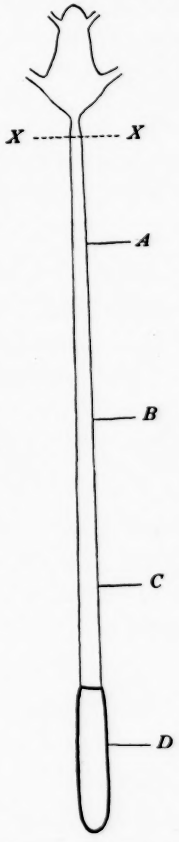


FIG. 2.—Diagrammatic outline indicating levels of incisions, of origin of grafts, and of implantation. Control incisions at levels *A*, *B*, and *C*. Distal grafts from donor level *A;* proximal grafts from donor level *C*. Implantation at levels *A*, *B*, and *C* and in a few experiments at *D*. Removal of distal hydranth approximately at level of broken line, *XX*.

mately the middle of the naked region of the stem (Fig. 2, *B*); the proximal level, 5–8 mm. distal to the distal boundary of the perisarc (Fig. 2, *C*). Since the ability of the stem to extend and contract decreases from the distal end proximally, the variation of level of graft in different individuals was greatest at the distal level.

Pieces for grafting were taken from two levels of the donors—distal and proximal (Fig. 2, *A*, *C*). For convenience the grafts from these two levels are designated, respectively, as "distal" and "proximal" grafts. For comparison of different levels of the stem as host and as donor, grafts were made in the following combinations of body level: distal on distal, proximal on distal, distal on middle, proximal on middle, distal on proximal, proximal on proximal. Two, or in some cases three, lots of ten each, that is, twenty or thirty cases of each, of these combinations of grafts were made with the host hydranth present, and the same number with removal of the host hydranth at the time of grafting—a total of two hundred and sixty grafts. In addition to these, controls were also prepared, consisting of stems like the host stems. In these a lateral incision was made at the desired level, as in the host stems, and the entodermal parenchyma was slightly crushed. Twenty or thirty of these controls were prepared for each level—distal, middle, and proximal—with the original hydranth present, and the same number with the hydranth removed—a total of one hundred and sixty.

In addition to this attempt to analyze the effect of body level of graft and of host on development of new axes from grafts and incisions, some other experiments were performed, viz., grafting of larger pieces than those used in the body-level series, grafting of two pieces in the same incision, grafting of sectors of hydranths undergoing reconstitution, grafting of pieces of manubria of fully developed hydranths, and grafting of aggregates of dissociated cells. These experiments were essentially exploratory in character and few in number.

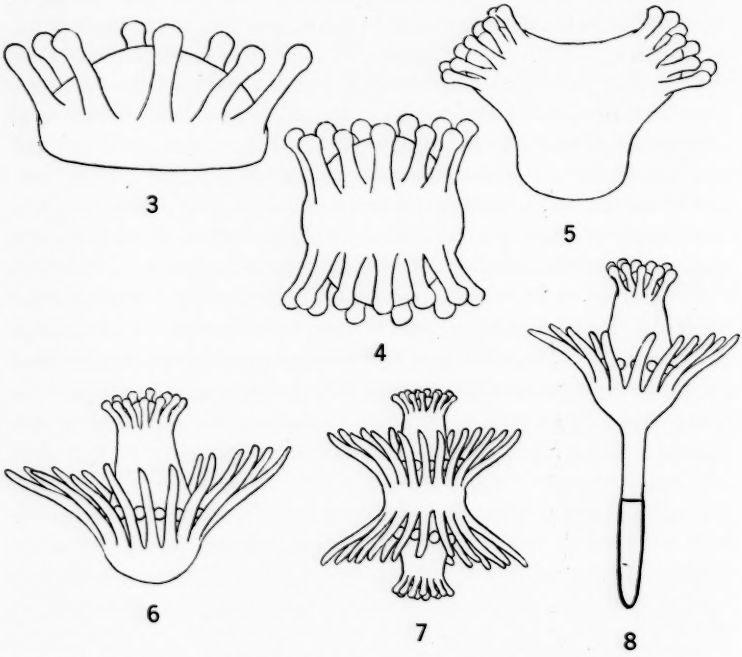
All figures except the outlines, Figures 1, 2, 27, are free-hand drawings of living individuals, but are diagrammatic as regards gonosomes or gonosome buds and numbers of tentacles. Perisarcal regions are indicated by a heavy line.

EXPERIMENTAL

Reconstitution of isolated graft pieces.—It has been shown in earlier papers that short transverse pieces of the stem of *Corymorpha* may give rise to a great variety of forms, ranging from hypostomes only through manubria, complete hydranths, hydranths with stems, to complete individuals. The partial forms consisting of the more distal hydranth regions may be unipolar, bipolar, bipolar-unipolar, or multipolar; and even the complete individuals may be unipolar or bipolar (Child, 1926*b*). Moreover, in the study of the development of aggregates of dissociated cells it was found that aggregates as small as 0.2–0.3 mm. in diameter in the spherical form might develop into any of the above-mentioned forms from a hypostome to a complete individual, depending on the scale of organization (Child, 1928*b*). The pieces used in grafting are intermediate in size between the short transverse pieces and the aggregates. If they reconstitute at all when isolated, it may be expected that they will give rise to the same range of forms. A few lots of the $\frac{1}{4}$ sectors of short pieces, such as were used for grafting, which reconstituted as isolated pieces, showed that this is the case. These sectors gave rise to the range of forms shown in Figures 3–8. In short, these pieces, like other small pieces, may give rise to one or more polarities; and in each polar axis the apical end and as much more of the body is present as the size of the piece, the scale of organization determined in each axis and the presence or absence of other developing axes permit. The frequency of unipolar, bipolar, and bipolar-unipolar partial forms (Figs. 3–5) is highest in pieces from the more distal levels of the stem and decreases proximally; and the frequency of complete individuals increases in the same direction, that is, the scale of organization (Child, 1928*b*, 1931) decreases from distal to proximal levels. In *Tubularia* a similar distal-proximal differential was noted by Driesch (1897). When the *Corymorpha* sectors remain undisturbed on the bottom of the container, unipolar or bipolar-unipolar forms ranging from hypostomes to complete individuals (Figs. 3, 5, 6, 8) develop more frequently than when their positions are changed.

The results of grafting.—When the $\frac{1}{4}$ sectors are grafted into lateral incisions in the stem, development of a new axis may or may not occur, according to conditions. If it does occur, anything from a

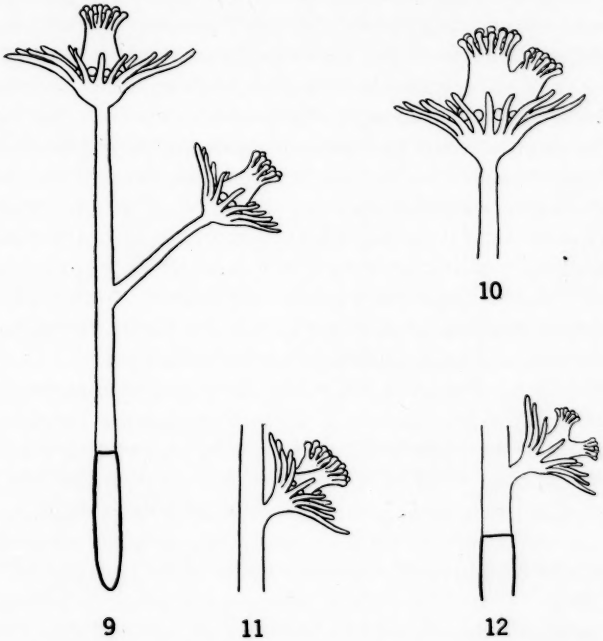
hypostome to a complete hydranth with a greater or less length of stem may develop, or occasionally, in the case of proximal grafts, a basal end. If anything more than the extreme apical portion of a hydranth develops, the graft itself constitutes more or less of the



FIGS. 3–8.—Forms which develop from isolated ¼ sectors similar to graft pieces. Drawn to about ten times the scale of following figures.

apical region of the new axis, the remaining parts developing from the tissue of the host. Figure 9 shows a new axis developed in this way (see also Child, 1929*a*, Figs. 3–10). In such cases the graft evidently acts as an "organizer" or dominant region, at least in most cases. The length of stem which develops during the usual laboratory life of the material depends on the level of implantation and apparently on the condition of the developing hydranth as indicated by its scale of organization and rate of development. If the graft is

made at a level which is included in the region of reconstitution of a hydranth, the new axis determined by the graft develops nothing more than a duplication of the portion of the hydranth distal to the level of implantation (Fig. 10; also Child, 1929*a*, Figs. 16–20). If the



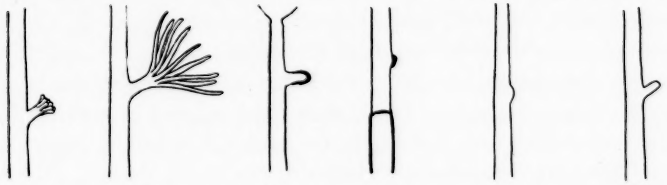
FIGS. 9–12.—Various forms of new axes determined by grafts. Figure 9, complete normal hydranth and stem. Figure 10, graft in area of hydranth reconstitution; determines only distal hydranth region. Figures 11 and 12, duplication of distal hydranth region in new axes determined by grafts.

level of implantation is slightly more proximal, the new axis may develop as a hydranth without stem (Child, 1929*a*, Figs. 21–23). In such cases no later development of more proximal regions has ever been observed. At still more proximal levels, the new axis may develop a greater or less length of stem (Fig. 9). In general the length of stem is greater the farther proximal the level of implantation. Apparently, the new axis develops primarily at most the parts corre-

sponding to the parts of the host body which are distal to the level of implantation, or, if its dominance is more effective than that of the host, it may perhaps develop parts representing levels slightly proximal to the level of implantation. The dominance of the graft tissue reorganizes the host tissue into these more distal parts but has no direct reorganizing action which will determine development of more proximal regions, because the cells of the host body already possess the organization characteristic of these levels and the new dominance does not change it. It is possible, however, that in cases like Figure 9, in which the new axis attains an advanced degree of development, a gradual increase in length may occur if the material remains in good condition and that separation of the two individuals might finally occur if the animals lived long enough. Such secondary growth of the new axis, so far as it occurs, is probably not a reorganization of the host tissue but a growth of the graft axis at the expense of the more proximal levels of the host body. So far, complete separation of host and graft axis has never been observed.

Duplications of more or less of the distal hydranth region are not infrequent in the development of the graft hydranths, but no case of duplication of the whole hydranth has been observed thus far. These duplications may occur at any level of implantation, and the plane of duplication may form any angle with the polar axis of the host (Figs. 11, 12). It was shown in the earlier paper that in some of these duplications both hypostomes were formed by the graft tissue (Child, 1929*a*, Figs. 17, 18, 22), while in others one hypostome develops entirely from host tissue (Child, 1929*a*, Figs. 20, 23); and also that anything from a hypostome to a hydranth with more or less stem may develop from an incision at the more distal levels of the stem without implantation of a graft. The controls of Tables I–III confirm and extend the earlier observations. These differences suggest that in some cases the graft develops as a bipolar (Fig. 4) or bipolar-unipolar form (Fig. 5) and in others as a unipolar form, with occasional development of another axis from the host tissue. This interpretation is supported by the fact that in all controls, that is, simple incisions without grafts (totaling more than two hundred cases, including the earlier experiments), only one duplication has been observed —and this involved only the extreme distal hypostome region.

In some cases the graft fails to develop anything more than a partial hydranth. In the great majority of cases this is the distal hydranth region, for example, a hypostome with perhaps more or less of the manubrium (Fig. 13). Apparently, such grafts are capable of little or no organizing action, either because of poor condition at the time of grafting or because they are inhibited after grafting. Occasionally, partial forms, like Figure 13, will slowly develop proximal tentacles and perhaps a short stem if kept long enough, that is, 10 days or more, or if the host hydranth be removed during the first few days following grafting. In most cases, however, the proximal parts



FIGS. 13–18.—Partial hydranths, basal regions, and outgrowths determined by grafts. Figure 13, hypostome and manubrium only; no further development. Figure 14, group of proximal tentacles. Figures 15 and 16, basal regions: Figure 13, proximal on distal; Figures 14, 15, and 16 proximal on proximal. Figures 17 and 18, outgrowths with structure of stem.

of the hydranth, if not present within 3–5 days after grafting, are not developed later.

Rarely (three cases among some five hundred grafts) a group of tentacles identical in structure and appearance with proximal, rather than distal, tentacles develops, with a short outgrowth proximal to them (Fig. 14). These forms may include some host tissue as well as the graft. In such cases mouth, hypostome, and manubrium are entirely absent, and proximal to the tentacle group there is no visible demarcation of hydranth and stem. Apparently, these are cases in which the graft attains only the physiological or metabolic level at which development of proximal tentacles but not the distal parts of the hydranth occurs. Evidently, the persistence of this condition is rare, for in all other cases the hydranth develops from the distal end proximally; and if any parts are absent, they are proximal rather

than distal. At present it seems probable that forms of this sort represent an arrest or inhibition of activation of the graft at a very early stage, before it has attained its maximum activity, or a failure on the part of the graft itself to progress beyond partial activation. In pieces undergoing reconstitution, similar groups of proximal tentacles also appear rarely and under conditions which suggest inhibition or failure for some other reason to undergo activation beyond an early stage.

Grafts of sectors from the proximal level (Fig. 2, *C*) sometimes give rise to basal regions covered with, and secreting, perisarc instead of developing as hydranths (Figs. 15, 16). These basal regions may develop from proximal grafts implanted at any level. In all cases thus far observed, they cease growth at an early stage and do not develop, beyond the size indicated in the figures, during the laboratory life of the animals. The point of chief interest as regards these structures is that they develop from grafts taken from a level somewhat distal to the perisarcal region of the stem, that is, the graft becomes more proximal in character than the level from which it came. Among the one hundred and ten proximal grafts of Tables I–III below, fifteen cases of development of basal regions occurred. A few of them which show some outgrowth, such as Figure 15, apparently involve host tissues to a slight extent, that is, the graft probably reorganizes host tissue to a slight extent in these cases as well as in hydranth development. Further discussion of these cases is postponed to a later section (see pp. 617–18).

The graft may also give rise to a small growth, often a mere protuberance, without any distinguishing characteristics of hydranth or base (Figs. 17, 18). In some cases these outgrowths are distinctly larger than the graft and appear to involve some host tissue, that is, even here the graft may determine some slight degree of reorganization, although the outgrowth appears to be nothing more than stem tissue.

And finally, the development of the graft may be completely inhibited or fail to occur because of conditions in the graft itself. Inhibition in such cases is not due to complete closure of the ectoderm of the host over the graft, for the graft often forms part of the surface of the stem even though it fails to develop. Unless the graft is pur-

posely implanted deep in the parenchyma, the host tissue rarely, if ever, closes completely over it; and in all cases of deep implantation observed thus far the graft has died without development or outgrowth.

Tables I–III.—These tables give the results of implantation of distal and proximal graft pieces at the three host levels—distal (Table I), middle (Table II), and proximal (Table III). Each table consists of three sections, the data of the controls being given in the first, those of distal graft pieces in the second, those of proximal graft pieces in the third. The first three vertical columns of the tables require no explanation. In the fourth column, headed "Distal Hydranth" the plus and minus signs for the different lots indicate the presence (+) or the absence (−) of the original hydranth of the host or control body. It will be observed that in half the controls and half of the transplantations for each combination of body levels the distal hydranth remains and that in half it is removed. The column headed "No Outgrowth" lists those cases in which the region of the graft showed no appreciable outgrowth beyond the normal contour of the stem. In all cases listed under this head, the graft was observed to be present. The sixth column, "Outgrowth," includes all cases in which some outgrowth occurred but never developed any characteristics of a hydranth, though occasionally it became a basal region. The number of basal regions developing is given for each lot in which they appeared. The seventh column, "Complete or Partial Hydranth," includes all cases in which a hydranth or partial hydranth developed in relation to the graft or the incision alone. The number of partial hydranths and duplications is given for each lot. The eighth column lists the few cases in which the graft was lost. The last two columns give, respectively, total percentages of outgrowths and of hydranths or partial hydranths in each series of experiments for each combination of levels with host hydranths, for each combination without host hydranths, and for the controls with, and those without, distal hydranths.

Grafts at the distal level.—Table I gives the data for controls with incisions at the distal level and for distal and proximal grafts at the distal level. In the controls no development occurs when the distal hydranth is present, but 56.7 per cent develop hydranths when the

distal hydranth is removed. The implantation of a distal graft at the distal level (second section) results in 70 per cent of outgrowths and only 10 per cent of hydranths when the host hydranth remains; but when the host hydranth is removed, 85 per cent develop hydranths. The proximal graft implanted at the distal level (third section)is less

TABLE I

CONTROLS (INCISIONS WITH INSERTION OF NEEDLE) AND GRAFTS AT DISTAL LEVEL OF STEM

Operation	Lot No.	No. of Cases	Distal Hydranth	No Outgrowth	Outgrowth	Complete or Partial Hydranth	Total Percentage of Outgrowth	Total Percentage of Complete and Partial Hydranths
Incision at distal level	18	10	+	10	.....	.....	0	0
	23	20	+	20	.....	.....		
	11	10	−	3	.....	7(1 partial)	0	56.7
	24	20	−	10	.....	10(1 duplicate)		
Graft; distal on distal...	9	10	+	2	6	2(1 partial)	70	10
	29	10	+	2	8	.....		
	10	10	−	2	.....	8	0	85
	30	10	−	1	.....	9(6 partial, 1 duplicate)		
Graft; proximal on distal.....	31	10	+	4	5	1	60 (10% bases)	5
	33	10	+	3	7(2 bases)	.....		
	32	10	−	3	2(1 base)	5(3 duplicate)	35 (25% bases)	45
	34	10	−	.....	5(4 bases)	4(1 duplicate)		

effective in determining a new axis: 60 per cent outgrowths and 5 per cent hydranths develop when the host hydranth is present, 35 per cent outgrowths and 45 per cent hydranths when the host hydranth is removed. Evidently, the distal graft at the distal level is much more effective in inducing hydranth development when the distal hydranth is removed than when it is present, and it is also more effective than the proximal graft and more effective than the controls in the absence of the distal hydranth. The proximal graft, on the other hand, even in the absence of the host hydranth, is somewhat less ef-

fective in inducing hydranth development than the corresponding control, though the difference, 11.7 per cent, is undoubtedly not large enough to be significant. In the absence of the host hydranth, however, 25 per cent of the proximal grafts give rise to basal regions. Taking both hydranths and basal regions together, the proximal graft appears to be somewhat more effective than the control in inducing definite development of some kind, though the difference, 13.3 per cent, is of doubtful significance. All three sections of Table I

TABLE II

CONTROLS (INCISIONS WITH INSERTION OF NEEDLE) AND GRAFTS AT MIDDLE LEVEL OF STEM

Operation	Lot No.	No. of Cases	Distal Hydranth	No Outgrowth	Outgrowth	Complete or Partial Hydranth	Graft Lost	Total Percentage of Outgrowth	Total Percentage of Complete and Partial Hydranths
Incision at middle level	17	10	+	10	.....	.....	...	0	0
	25	20	+	20	.....	.....	...		
	3	10	−	6	.....	4	...	0	26.7
	26	20	−	16	.....	4	...		
Graft; distal on middle..	6	10	+	3	5	2	...	50	30
	15	10	+	.....	5	4(1 duplicate)	1		
	7	10	−	1	1	8	...	15	80
	16	10	−	.....	2	8(1 partial)	...		
Graft; proximal on middle.....	1	10	+	5	4	1	...	55	5
	13	10	+	1	7	.....	2		
	2	10	−	2	1	7(1 duplicate)	...	15	70
	14	10	−	1	2	7(1 partial)	...		

agree in showing clearly that the frequency of hydranth development is much higher, both in controls and in grafts, when the distal hydranth is absent than when it is present. Evidently, the distal hydranth as the dominant region of the host and control body exerts in some way a highly effective inhibiting action on hydranth development both in the control and the grafts. If the data of all three sections of Table I are combined, the hydranth frequency is 4.3 per cent in the presence of the distal hydranth and 61.4 per cent in its absence and for the two series of grafts, 7.5 per cent when the host hydranth is present, 65 per cent when it is absent.

Grafts at the middle level.—It is evident from Table II that both

distal and proximal grafts are much more effective than the controls in the absence of the distal hydranth. In the presence of the distal hydranth the distal graft is more effective (30 per cent hydranths) than the proximal (5 per cent hydranths), but in the absence of the distal hydranth there is little difference between distal and proximal grafts (distal 80 per cent, proximal 70 per cent). As in

TABLE III

CONTROLS (INCISIONS WITH INSERTION OF NEEDLE) AND GRAFTS AT PROXIMAL LEVEL OF STEM

Operation	Lot No.	No. of Cases	Distal Hydranth	No Outgrowth	Outgrowth	Complete or Partial Hydranth	Graft Lost	Total Percentage of Outgrowth	Total Percentage of Complete and Partial Hydranths
Incision at proximal level	21	20	+	20	.....	.....	...	0	0
	22	20	−	20	.....	.....	...		
Graft; distal on proximal	35	10	+	1	7	1	1	60	10
	37	10	+	4	5	1	...		
	36	10	−	1	1	8(1 duplicate)	...	10	55
	38	10	−	6	1	3	...		
Graft; proximal on proximal	27	10	+	.....	10(3 bases)	.....	...	56.7 (13.3% bases)	0
	39	10	+	5	5	.....	...		
	41	10	+	8	2(1 base)	.....	...		
	28	10	−	2	6	2	...	43.3 (13.3% bases)	33.3
	40	10	−	5	3(2 bases)	2	...		
	42	10	−	.....	4(2 bases)	6	...		

Table I, the dominance of the distal hydranth is clearly evident, both in the controls and the grafts. Combining all three sections of the table, 10 per cent of hydranth development occurs in the presence of the distal hydranth, 55.7 per cent in its absence. Taking the grafts alone, 17.5 per cent develop hydranths with host hydranth present, 77.5 per cent with host hydranth removed.

Grafts at the proximal level.—The differences between controls and distal and proximal grafts in Table III are in the same direction as in the other tables. The incision alone does not determine develop-

ment in any case. The table shows that in the absence of the distal hydranth the distal grafts are more effective than either the controls or the proximal grafts, and that both distal and proximal grafts are more effective when the distal hydranth is absent than when it is present. The totals for all grafts at the proximal level are: 4 per cent in the presence and 38 per cent in the absence of the host hydranth. The controls show no hydranth development in any case. The percentages of hydranth development in the absence of the distal hydranth are lower in Table III than in Tables I and II. This is probably due in part to the fact that almost all of the grafts at the proximal level were made late in July, when the animals were becoming stunted and were not in as good condition as earlier in the month, apparently on account of the rising water temperature in the shallow bay, connected with the ocean by only a single narrow inlet, which is their habitat.

Certain general results.—Comparing the controls of the three tables, it appears that the incisions are most effective in determining hydranth development at the distal level (56.7 per cent), less so at the middle level (26.7 per cent), and not effective at the proximal level. The total frequency of development of complete and partial hydranths from grafts at all levels in the absence of the host hydranth is for distal grafts 73.3 per cent, and for proximal grafts 45.7 per cent plus 12.9 per cent basal regions. Earlier experiments (Child, 1929*a*, Table I, p. 346, Table II, p. 349) with removal of host hydranths gave for distal grafts (distal on proximal, thirty animals) 83.3 per cent hydranths, for proximal grafts (proximal on proximal, twenty animals; proximal on distal, thirty animals) 72 per cent, for controls at the distal level 33.3 hydranths, and for controls at the proximal level none. In these earlier experiments larger pieces—even whole transverse pieces—were used for grafts in some cases, so that the hydranth frequencies are probably not strictly comparable to those of Tables I–III above; nevertheless, the differences with respect to level of stem are in the same direction in both.

Data on larger numbers of individuals would undoubtedly change the percentages somewhat, and probably winter material would give somewhat different percentages, for at that time the animals are in better condition, attain larger size, and live much longer in the

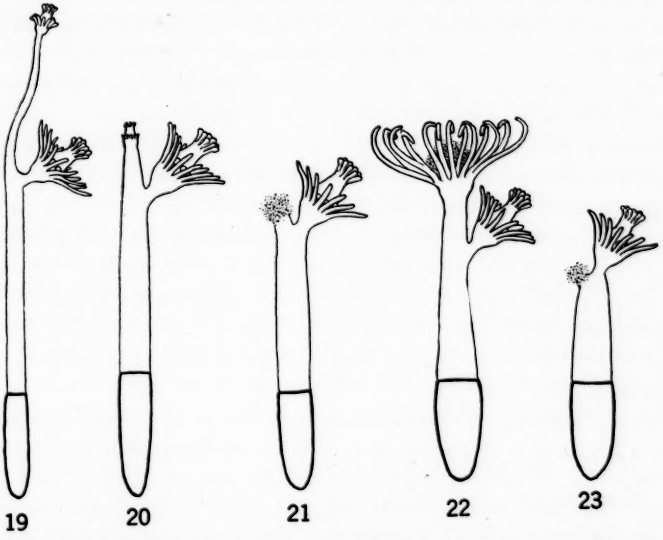
laboratory than in summer. But the differences to which attention has been called are so large and so consistent in the three tables that there can be no doubt of their physiological significance.

The number of partial hydranths and duplications is too small to permit any conclusions as to their distribution, but it may be pointed out that of the ten partial hydranths and nine duplications of the tables only one of each occurs in the controls. Probably most of the partial forms represent development of the graft with little addition of host tissue. The duplications involve only the more distal portions of the hydranth and probably result in most, if not all, cases from the development of two, or occasionally three, groups of active cells from the graft instead of one; but these groups are so close together that in later development their effects are combined and determine only a single proximal region. In some cases duplication may result from development both from graft and from incision.

Basal regions develop from proximal grafts at both distal and proximal levels. Since they develop infrequently at best, their absence from the middle level is probably without significance. Further experiments with grafts from various proximal levels, both in the perisarcal region and distal to it, will undoubtedly assist in determining the conditions which bring about development of basal regions instead of hydranths.

Relations between graft hydranth and host hydranth.—It is evident from the data of Tables I–III that the host hydranth, when present, frequently prevents the development of a new axis from the graft, and that at the distal and middle levels it also prevents hydranth development in the controls. In view of this and other facts concerning the dominance of the hydranth region, it is of interest to inquire whether there is any evidence that the graft hydranth ever attains dominance over the host hydranth. Evidently the graft, unless inhibited, dominates and determines the reorganization of adjoining host tissues; but the question now raised is whether the fully developed graft hydranth is able to become dominant over more distant parts of the host body. In transplantations in which the host hydranth is removed at the time of grafting and the graft hydranth develops rapidly, it often appears to retard the development of the host hydranth. Forms like Figure 19 are not uncommon. This was

a distal graft at the middle level with removal of the host hydranth. The graft hydranth, which of course began to develop at approximately the same time as the reconstituting host hydranth, is much larger and more advanced than the latter; and the stem distal to the graft is slender and weak. In this case the distal hydranth, although



FIGS. 19–23.—Relations between new and old axes. Figures 19–21, partial dominance of graft hydranth over reconstituting host hydranth and distal levels of host stem. Figures 22 and 23, apparently more complete acclimation of physiologically younger graft axis to laboratory conditions than of original host hydranth and distal stem levels.

evidently retarded, continued to grow slowly and finally became as large as the graft hydranth.

Another case, also a distal graft at the middle level with removal of host hydranth, is shown in Figure 20, 5 days after grafting. The graft hydranth is large and in good condition, while the reconstituted host hydranth and the stem distal to the graft are much reduced. Three days after grafting, the graft hydrant and the host hydranth were about the same size; but during the next 2 days the graft hydranth increased and the host hydranth decreased in size. Fig-

ure 21 shows the same case 3 days later. The host hydranth and almost all of the stem distal to the graft have disintegrated. In this case the dominance of the graft hydranth seems to be complete.

A greater or less degree of graft-hydranth dominance may appear with grafts at any of the three levels, but seems to be most frequent with distal grafts on the middle level. In grafts at the proximal level, the distance is perhaps too great for the graft hydranth frequently to attain dominance over the distal hydranth. In grafts at the distal levels, the graft not infrequently lies within the region of host-hydranth reconstitution and develops only a partial hydranth; but when it is proximal to the region of hydranth reconstitution of the host, no case of complete dominance of the graft hydranth over that of the host has been observed. Often host and graft hydranths are of the same size and develop at the same rate without any appreciable effect on each other, as if each neutralized or compensated the effect of the other. Occasionally, the graft hydranth seems to inhibit the host hydranth to a greater or less extent, but the graft hydranth may remain small or partial, apparently inhibited by the host hydranth.

After a week or two in the laboratory in summer, even in running water, the larger, older individuals of *Corymorpha* begin to degenerate and die. Young or reconstituting animals live longer. The hydranth dies first in almost all cases, and death progresses proximally along the stem but is preceded by shortening and thickening and loss of contractility. In experimental individuals with the original host hydranth and a lateral-graft hydranth the host hydranth, with rare exceptions, degenerates and dies while the graft hydranth is still in good condition. A case of this sort is shown in Figure 22: the host hydranth has disintegrated almost to the level of the proximal tentacles, and these show incurving tips, a condition often observed before degeneration. The graft hydranth (distal graft on middle level) is still in good condition, but the stem region distal to it is shortened to a much greater degree than that proximal. Figure 23 shows the same individual 4 days later. The original host hydranth and the distal half of the stem have disappeared, except for the disintegrating stump; but the graft hydranth and the stem proximal to it are still in good condition. These cases, which are common, ap-

pear, at first glance, to indicate dominance of the graft hydranth over the original-host hydranth; and it may be that such dominance plays a part in the result. There is, however, another factor. As already noted, physiologically young animals, that is, those which develop from eggs or are reconstituted from small pieces in the laboratory, live much longer than large, old animals—often two or three times as long, even without special feeding. Apparently the young organism is able to acclimate to laboratory conditions to a greater degree than the old. In cases like Figures 22 and 23 the original host hydranth is physiologically older than the graft hydranth. Even if the host hydranth lives as long as the hydranths of other unoperated individuals, it usually dies several days before the graft hydranth. It is not necessary, then, to assume that the earlier death of the original host hydranth is due to dominance of the graft hydranth; it may be due merely to the difference in physiological condition of the two hydranths. On the other hand, it appears probable that the dominance of the graft hydranth is a factor in maintaining the stem proximal to it in good condition for a longer time than is usual in unoperated individuals of the same size. In this region of the stem the dominance acts in the normal direction, not in reversed direction, as in the region distal to the graft.

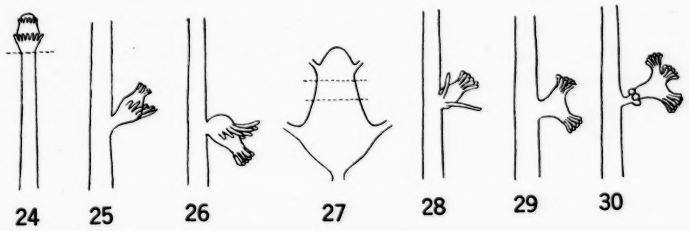
Supplementary experiments.—During the course of grafting in relation to body level, various other grafts were made from time to time, chiefly by way of exploration of some of the further possibilities. The results of some of these experiments are briefly stated below because they are of interest as supplementing the tabulated data.

1. Two separate $\frac{1}{2}$ sectors of pieces 1–2 mm. in length were implanted at the proximal level in stems from which the hydranths were removed. The result was ten (100 per cent) graft hydranths—eight normal single hydranths and two with duplication of the distal part of the manubrium and hypostome. In spite of the fact that two separate pieces, each twice as large as the usual graft, were implanted, there is no indication in any case of duplication of the more proximal hydranth regions, and duplication of the distal regions occurs in only two cases. This result indicates that the original polarity and structure of the grafts plays very little, or no, part in determining the new axis.

2. Single ¼ sectors of pieces 1–2 mm. long from the region of the holdfast buds (Fig. 2, *D*), that is, several millimeters proximal to the distal boundary of the perisarc, were implanted at the proximal level of the naked stem (Fig. 2, *C*) in ten individuals with removal of the host hydranth. The result was: three no outgrowth, three bases, three hydranths with basal structures just proximal to the hydranth body, one hydranth with a second manubrium beside it, and one short outgrowth with two groups of proximal tentacles. Half of these grafts developed basal regions, but two of these developed hydranth as well as base. Apparently, the frequency of development of a base from the graft increases as the donor level becomes more proximal, but these grafts also give rise to hydranths; in other words, the development of bases does not result from inability to develop hydranths but from some factor in physiological condition which may or may not determine the result.

3. Sectors of reconstituting hydranths were implanted at the proximal level of the naked stem. The hydranths and 2–3 mm. of the stem were removed from the donors, and 48 hours later the developing hydranths, at the stage indicated in Figure 24, were removed and cut into longitudinal ¼ pieces which were used as grafts. The hydranths were also removed from the host stems 48 hours before grafting; consequently, the host hydranths were at approximately the same stage of development as the donor hydranths from which the graft pieces were taken. These developing host hydranths remained on ten stems (Lot A) and were removed from ten (Lot B) when the grafts were implanted. In Lot A the grafts determined seven normal hydranths and three small outgrowths; in Lot B, four normal hydranths, three partial hydranths consisting of manubria with distal tentacles, and three small outgrowths. In the earlier stages of this development the graft hydranths showed in their asymmetry indications of their origin from ¼ sectors of a hydranth (Figs. 25, 26). The circle of distal tentacles was complete, but proximal tentacles were present only on about half the circumference and were longest in the middle region of that half. Later, all cases which developed proximal tentacles at all became normal symmetrical hydranths and induced the development of stems. Whether, or to what extent, the host tissue took part in the completion of the hydranth

body was not determined, but the stems undoubtedly developed from host tissue. The different orientations of the hydranths in earlier stages indicated that the grafts retained their original polarity. The three cases of Lot B which gave rise only to partial forms represent less longitudinally than the original graft. The fact that these partial forms developed in Lot B from which the reconstituting distal hydranths were removed at the time of grafting makes it probable that they are not due to inhibition by the host hydranth but rather to local conditions, for example, injury of the proximal region of the graft in preparation or position of the graft or perhaps



FIGS. 24–30.—Grafts of parts of hydranth. Figure 24, stage of reconstitution at which ¼ hydranth sectors were used as grafts. Figures 25 and 26, new axes from ¼ hydranth sectors. Figure 27, outline of hydranth; piece of manubrium between broken lines used as graft. Figures 28–30, new axes determined by the manubrium grafts.

reorganization. How much reorganization of the hydranth region at the stage of reconstitution used in the experiments is possible is not at present known. In nine of the eleven cases with normal graft hydranths, the host hydranth and the host stem distal to the graft level disintegrated within 9 days, while the graft hydranth and the stem proximal to it were still in good condition. In the three cases with manubria only, further development did not occur, the manubria decreased in size, and in two of them the tentacles underwent resorption while the host hydranths remained in good condition. In the third case, the graft manubrium attained about the same size as that of the host hydranth, and the two underwent degeneration at about the same time.

4. In five individuals a piece consisting of the middle region of the manubrium of a fully developed hydranth with a cut surface at each

end (Fig. 27) was grafted at the middle level of the stem. Of the five, two developed, as in Figure 28, into hydranths with normal distal tentacles but only two or three proximal tentacles and no induced stem. Two developed into bipolar hypostomes with a unipolar more proximal portion but no proximal tentacles (Fig. 29). One gave rise to the form shown in Figure 30, with three hypostomes and a unipolar proximal region bearing a few gonosome buds but no proximal tentacles. The two cases like Figure 28 evidently represent unipolar development of the graft with partial development of the proximal hydranth regions. The three cases like Figures 29 and 30 probably represent bipolar development of the graft with induction proximally of the gonophore level in one case. So far as known, pieces of the manubrium, when isolated, do not give rise to more proximal regions of the hydranth. This being the case, three of the grafts have evidently determined the development of more proximal regions from the host tissue. Further experiments with parts of the developing and fully developed hydranth are desirable.

5. Grafts of aggregates of dissociated cells were made in ten cases. Stems were dissociated as described in an earlier paper (Child, 1928*b*); and aggregates somewhat less than 1 mm. in diameter were used as grafts as soon as they had attained a rounded form and sufficient coherence of the cells to permit the necessary handling, that is, 6 or 7 hours after dissociation. They were implanted at the proximal level of the naked region (Fig. 2, *C*), and the host hydranth was removed. In three cases a hydranth or hydranth and stem developed. Hydranth development from a simple lateral incision at the proximal level has not been observed in any case in the controls of Table III above and in similar controls for earlier experiments (Child, 1929*a*, Table I), a total of sixty individuals. Hydranth development in 30 per cent of these aggregate grafts leaves little doubt that the graft induced the development. Since the aggregate is able to develop anything from a partial hydranth to a complete individual (Child, 1928*b*), this result is to be expected. Apparently in some, perhaps in all, cases that showed no development, the graft was completely inclosed by the host tissue and took no part in closure of the incision. Further experiment is necessary to determine whether a graft can ever determine development if it is completely inclosed.

DISCUSSION

Graft and wound tissue as dominant regions.—Attention has been called in earlier papers to the fact that the graft or the region of the healed incision may become the dominant region or "organizer" in the development of a new axis (Child, 1927*c*, 1929*a*, 1929*b*). As a further contribution to the subject, the data of Tables I–III above require consideration. The tabulated data show that at the distal and middle stem levels axiate development—partial hydranth, hydranth, and stem—may result from a simple lateral incision without graft, provided the original distal hydranth be removed. They also show that both with and without the original distal hydranth the frequency of axiate development is, in general, higher when a graft is present than in the corresponding control with incision. In the case of hydranth development determined by a graft, the graft constitutes the apical end and more or less of the distal region of the hydranth, the remainder of the hydranth body and the stem developing from the host tissues (Child, 1929*a*, Figs. 3–14, 16–18, 20–24). Evidently, in such cases the graft acts as an "organizer" or dominant region (Child, 1929*b*). In some cases, however, a hydranth or partial hydranth may develop entirely from host tissue, even though a graft is present. In cases of duplication, for example, one component may have the graft at its tip while the other develops from host tissue (Child, 1929*a*, Figs. 20, 23); and in one case observed, two partial hydranths developed from host tissue with the graft lying between them (Child, 1929*a*, Fig. 19). Such development of a distal hydranth region from host tissue when a graft is present has been observed thus far only in cases of proximal grafts at the distal level; but since hydranths may develop from incisions without grafts at the middle level, it may occasionally occur there also. In any case, it is merely development from the region of growth which results from the incision; and if the graft does not dominate the whole area, or does not become dominant at all, a new axis or new axes may develop entirely from the host tissue, as if no graft were present.

The dominance or organizing action of the graft is obviously associated with its great capacity for rapid reconstitution. Even when completely isolated, it may develop into anything from a hypostome to a complete individual, depending on the scale of organization.

The graft implanted in the host stem is undoubtedly in a more favorable environment than when isolated in water; consequently, its organization on a large scale may be expected, that is, it is likely to give rise only to the apical region of a hydranth (Child, 1928*b*). This, however, is evidently the primary dominant region of the hydranth; and since host tissue is present, the other parts of the hydranth and the stem develop through the reorganization of that tissue as determined by the dominant region.

The fact that a hydranth may develop from the region of increased physiological activity and growth which results from an incision seems to indicate that a dominant region or "organizer" is primarily not a specific organ or organ complex but is a region which is more intensely active physiologically than the regions about it. In the grafts neither the polarity nor any other feature of the organization can be regarded as responsible for the dominance and determining action of the graft. Single $\frac{1}{4}$ sectors, $\frac{1}{2}$ sectors, whole transverse sections, two pieces grafted in the same incision, sectors of reconstituting hydranths, and transverse sections of the manubrium of fully developed hydranths may act as dominant regions and determine development of single normal hydranths and stems.

Apparently, there is a threshold of cellular activity for hydranth development, and the activity resulting from the incision alone attains this threshold most frequently at the distal level (56.7 per cent), less frequently at the middle level (26.7 per cent), and not at all at the proximal level. But even at the proximal level, cellular activity may be brought above the level at which hydranth development occurs, by a lacerated incision (Child, 1927*c*). In view of all the facts, there seems to be no escape from the conclusion that organization of a hydranth in *Corymorpha* is initiated by a localized region of cellular activity or metabolism which attains a certain threshold of degree or duration, or both, and also a certain level or intensity in relation to regions about it. In other words, there appears to be a relative, as well as an absolute, threshold for the initiation of hydranth organization; and, so far as *Corymorpha* is concerned, the dominant region or organizer in its simplest terms is apparently nothing more than a localized region in which the cellular activity is above both thresholds. Probably any cell mass in which this condi-

tion exists and persists for a sufficient length of time is able to act as a dominant region.

The development of basal ends from grafts.—In a few cases grafts from the proximal level develop basal ends with perisarc (Figs. 15, 16). The tables show fifteen cases of basal development—seven from proximal on distal (Table I), eight from proximal on proximal (Table III). As already noted, some of these are definite outgrowths, while others are little more than a small area covered with perisarc. These bases possess a certain interest because the proximal grafts are taken from the naked region distal to the perisarc and in the intact animal do not give rise to perisarc. In these cases the graft becomes more proximal or basal in character than it was orginally. What conditions determine the development of these basal regions instead of hydranths is uncertain, but it is probable that they represent a slight degree of reorganization of the graft and development under the dominance of the host rather than as a dominant region itself. The proximal region of the *Corymorpha* stem is apparently a secondary gradient opposite in direction to the hydranth-stem gradient (Child, 1926*a*) and in the fully developed individual of course possesses a metabolism different in character in some respects from that of the primary gradient. The development of a proximal graft into a basal region may mean that the activity of the graft has been slightly accelerated, but within the limits of the proximal gradient, so that no extensive reorganization has occurred; consequently, development takes place under the dominance of the host body into a region slightly more proximal in character than the region of origin of the graft. Apparently, in order to develop as the apical region of a hydranth, a piece of stem must lose its original organization and become more or less completely embryonic in character. The proximal grafts may undergo this change and become the dominant regions of new hydranth axes; but if they do not attain a sufficiently high level of activity to isolate them physiologically from the dominance of the host, they develop as subordinate parts, either some level of the naked stem or, with a slight change in condition, as basal ends.

The question whether, or to what extent, these basal structures are dominant regions is also of interest. Figure 15 represents the maximum outgrowth observed in development of basal ends. This out-

growth was considerably larger than the graft when implanted and apparently developed in part from the host tissue. Vital staining of the graft is probably necessary for complete certainty as regards this point. In cases like Figure 16 there is certainly no dominance or organizing action on host tissue. The system of holdfast buds which arises in the secondary proximal gradient shows very definite relations of dominance and subordination of short range (Child, 1928*a*). Since this is the case, a basal region developing from a graft may be expected, under favorable conditions, to develop a short-range dominance; but the reorganization of host tissue cannot proceed far in any case because the proximal gradient is a secondary, subordinate gradient.

The development of indefinite outgrowths from grafts.—In these cases the graft undergoes little or no reorganization but brings about more growth than the incision alone. These outgrowths possess the structure of the stem and always remain of small size. In their maximum development they may represent development of a new stem axis (Fig. 18) and may perhaps attain some slight degree of dominance over adjoining regions of the host stem, so that the proximal region of the outgrowth develops from host tissue. This dominance and induced development has been observed only occasionally in these stem outgrowths. Usually the outgrowth is a mere protuberance with no indication of dominance.

The dominance of the distal hydranth.—Perhaps the most striking feature of the tables is the effect of the original distal hydranth in preventing hydranth development, both from grafts and from incisions. When the distal hydranth is removed at the time of grafting or incision, the frequency of lateral hydranth development is greatly increased in every series of experiments in all three tables, with the exception of the proximal incisions (controls, Table III) from which no hydranths develop even when the distal hydranth is removed. Evidently, the dominance of the distal hydranth is highly effective over the whole length of the naked stem region, not merely in preventing budding in nature but in preventing the development of hydranths from grafts and incisions. The tables show that the presence of the distal hydranth does not prevent the development of the small indefinite outgrowths with stem structure, that is, it does not pre-

vent the development of the graft as a subordinate stem region but only its development as another dominant hydranth region. When the distal hydranth is present, the cells of the graft and the region of incision are acted upon by correlative conditions which tend to maintain their characteristics as cells of the stem; and if these factors are sufficiently effective, the cells are prevented from undergoing the activation necessary for attainment of the threshold of hydranth development and establishment of a new gradient, and only healing of the incision or the small stem outgrowth of the graft occurs.

It is not necessary, however, to regard the dominance of the distal hydranth as exerted by means of a direct or positive inhibition of any sort. It is much more probable that the action of the dominant region on other parts is of the nature of stimulation in general and that it merely determines that the parts affected by it shall function as certain parts of the body. In other words, it is merely the most important factor in the intra-organismic environment of these cells or parts. In order to develop a new hydranth axis, the graft must lose its characteristics as a particular region of the stem and begin the development of a new individual with the organization of a new apical end, that is, a new dominant region. Whether or not this development takes place depends on the relation between the dominance which tends to maintain or restore the pre-existing condition in the stem, and on the local conditions which tend to induce activation, dedifferentiation, and growth.

The number of cases of development of basal ends from grafts is not sufficient to show clearly whether the presence of the host hydranth has any effect on the frequency of this development. From the grafts proximal on distal (Table I), only two bases develop when the host hydranth is present, and five when it is absent; but in the grafts proximal on proximal (Table III), four bases develop in the presence and four in the absence of the host hydranth. These data, together with the fact that only proximal grafts give rise to basal ends, indicate that the graft is a more important factor than the dominance of the distal hydranth in determining development of basal ends.

Hydranth-frequency in relation to origin of graft.—Examination of Tables I–III shows that at all three levels of implantation the graft

from the distal donor level gives a higher frequency of hydranth development than the graft from the proximal donor level. In the presence of the host hydranth the differences are mostly slight—5 per cent in Table I, 25 per cent in Table II, 10 per cent in Table III—but are all in the same direction. When the host hydranth is removed, the differences are 40 per cent in Table I, 10 per cent in Table II, and 22.7 per cent in Table III, also all in the same direction. Evidently the graft from the distal donor level is more effective in determining hydranth development than that from the proximal level. This difference is quantitative, not qualitative; and the only justifiable conclusion is that it is due to a quantitative difference in physiological condition between distal and proximal stem levels. If a physiological gradient is present in the stem, such a difference in effectiveness of grafts from different levels is to be expected. The graft from the proximal donor level is undoubtedly from the lower levels of the secondary proximal gradient, at least in some cases; but it always represents a gradient level below that of the distal stem region.

Hydranth frequency in relation to level of implantation or incision.—In the controls of Tables I–III hydranth development does not occur when the original distal hydranth is present; but in its absence the frequency is highest (56.7 per cent) at the distal, lower (26.7 per cent) at the middle, and zero at the proximal level. The absence of hydranth development at the proximal level is not due to inability of the cells to develop a hydranth, for hydranth development may be induced even at this level by a lacerated incision (Child, 1927*c*). Evidently, the cellular activity following a simple incision attains the threshold for hydranth development most frequently at the distal, less frequently at the middle, and not at all at the proximal level; but the greater or more persistent activity following the lacerated incision may bring even the proximal cells to the hydranth threshold. These different frequencies of hydranth development following incision at different levels point unmistakably to the existence in the stem of a quantitative physiological gradient of some sort.

The data on frequency of hydranth development following implantation of grafts at the three levels are complicated by certain factors. First, even when a graft is implanted, the hydranth may

sometimes develop from the incision without the dominance of the graft. Thus far, such development has been observed only at the distal level of implantation, and only rarely there; but the frequencies of hydranth development from grafts in the tables, particularly at the distal level, may include some cases of this sort. Second, grafts from the proximal donor level sometimes develop basal ends instead of hydranths both at distal and proximal levels, but the data do not show what determines this difference in behavior of grafts of similar origin implanted at approximately the same level. Third, in the present series of experiments the grafts at the proximal level were almost all made late in the season, when the animals were somewhat stunted and not in as good condition as earlier. The hydranth frequencies of Table III are low, as compared with those of Tables I and II, and considerably lower than those of distal on proximal and proximal on proximal of earlier experiments (Child, 1929*a*, Table I), perhaps because of the poor condition of the material. Because of these complicating factors, the percentages of hydranth development from grafts at different levels are not fully comparable; but further analysis brings to light facts of interest.

Comparing the effectiveness of grafts and controls in determining hydranth development, it appears that in the absence of the original distal hydranth the graft is, in general, more effective than the incision at the same level. The differences in percentages of hydranth development between the grafts at different levels and the corresponding controls are as follows: distal on distal, 28.3 per cent increase; distal on middle, 53.3 per cent increase; distal on proximal, 55 per cent increase; proximal on distal, 11.7 per cent decrease; proximal on middle, 43.3 per cent increase; proximal on proximal, 33.3 per cent increase. From these figures it appears that the frequency of hydranth development is increased over that of the control by the implantation of a graft to a greater degree at the middle and proximal levels than at the distal level. In fact, with the graft proximal on distal the hydranth frequency is somewhat below that of the control. These differences in effectiveness of graft and control incision in determining hydranth development also indicate the existence of a physiological differential or gradient, at least between distal and middle levels of the stem. The absence of any marked

difference between middle and proximal levels is probably due in part to the poor condition of the material used for grafting at the proximal level. In earlier experiments on material in better condition (Child, 1929*a*, Table I) the percentage increase in hydranth development from grafts at the proximal level over controls was: distal on proximal, 83.3 per cent; proximal on proximal, 45 per cent. Using these instead of the data of Table III above, the increases over controls for grafts of distal origin are: distal on distal, 28.3 per cent; distal on middle, 53.3 per cent; distal on proximal, 83.3 per cent. For grafts of proximal origin they are: proximal on distal, −11.7 per cent; proximal on middle, 43.3 per cent; proximal on proximal, 45 per cent. The effectiveness of the distal graft, as compared with that of the controls, increases from distal to proximal levels, and that of proximal grafts from distal to middle. If the basal ends which develop from proximal grafts are included, as well as the hydranths, the percentages of increase for proximal grafts, as compared with controls, become: proximal on distal, 13.3 per cent; proximal on middle, 43.3 per cent; proximal on proximal, 46.6 per cent according to data of Table III above, or 58.3 per cent with data of earlier experiments. These comparisons indicate that the lower the gradient level of implantation the greater the effectiveness of a graft of a given level of origin as compared with the control incision. It may be noted, however, that the proximal level is probably in the secondary proximal gradient in some cases and that this may be a factor in determining that the percentages of hydranth development from proximal grafts at middle and proximal levels do not differ very greatly. The level of origin of the proximal graft determines that in some cases it gives rise to basal ends instead of hydranths, so that its full effectiveness in determining development as compared with the control appears only in the total percentages of hydranths plus basal ends as given above. Further experiment with slight differences of level of grafting in the proximal region are desirable.

The polarity of the graft in relation to the new axis.—Apparently, the original polarity of the graft is not an important factor in its action as a dominant region. No attempt at orientation of the graft was made in any case, and it can scarcely be supposed that in all, or almost all, the grafts which determined hydranth development the

orientation was such that the original polarity of the graft corresponded to that of the new axis. It has been shown that in short pieces of the *Corymorpha* stem the original polarity is very readily altered or even obliterated by environmental conditions (Child, 1927*a*, 1927*b*) and the pieces used for grafting are much smaller than the isolated pieces of the earlier experiments. In so far as it is concerned in determining a new polarity, the graft is apparently nothing more than a group of active cells which raise the physiological or metabolic level of the cells about them above the threshold for hydranth development. The new gradient develops like the gradient of a bud in other Hydrozoa and many other cases of development of new axes of whole organisms or parts. The graft represents a localized active region; and, as in other cases, this region is not sharply bounded, but a radial gradient with decreasing activity evidently extends in all directions from the central most active region. As growth begins, this gradient becomes directly visible in the decreasing rate of outgrowth in all directions about the most actively growing central region, which is usually the graft itself, if a graft is present. As a result of this differential growth, the gradient which was originally radial becomes longitudinal, with its high end at the tip of the outgrowth. It is possible that some of the eight cases of duplication of the distal hydranth region following grafting represent development of a new axis from each end of the graft; but since seven of the eight occur at the distal and middle levels, one of the two axes may arise from the tissue closing the incision.

The cases described above (p. 611) in which two $\frac{1}{2}$ sectors instead of one $\frac{1}{4}$ sector were implanted in the same incision also indicate that in grafts of stem pieces the original polarity of the graft has little, or nothing, to do with the development of the new axis. Hydranths developed from all ten of these grafts; of these, seven were normal single hydranths, and in the other three duplication was limited to the hypostome and distal region of the manubrium. In the seven single hydranths no trace of the original polarities of the two grafts appeared, and in the earlier stages the outgrowth was single and the gradient evidently arose in the same way as with a single graft.

In the three cases of hydranth development after grafting an aggregate of dissociated cells, the grafted aggregate, or part of it, ap-

parently becomes a dominant region and determines hydranth development. This result, if confirmed by further experiment, constitutes still further evidence for the view that a region or cell mass may become dominant and determine axiate development without possessing any particular pre-existing organization. In the case of hydranth development from the tissue resulting from an incision without graft, the new polarity must arise in some way in the group of cells concerned. The gradient of the new axis arises in the region of the closed incision in the same way as about the graft. There is not the slightest evidence to indicate that the polarity of the new axis, whether determined by a graft or by an incision, is primarily anything more than this gradient; and, on the other hand, many different lines of evidence favor the view that polarity does originate in the gradient and that organization is the result, not the cause, of the gradient. There is apparently nothing but personal opinion in support of other views.

The results of grafting pieces of reconstituting hydranths indicate that these pieces usually—perhaps always—retain their original polarity and develop into a hydranth with relatively little reorganization. In the case of the transverse pieces of the manubrium, the frequent duplication of distal hydranth regions suggests bipolar development, that is, development of a distal region from both ends of the piece. These duplications from pieces of manubrium are apparently not very effective in determining development of more proximal regions. In some of them there is a common unipolar part proximal to the bipolar structures (Figs. 29, 30), and in one case (Fig. 30) this gives rise to gonophore buds, but none of them develop proximal tentacles. When unipolar development of these manubrial pieces occurs, a normal hydranth may result, though the proximal tentacles are at first few in number (Fig. 28). Further experiments on the polarity and dominance of grafts of parts of hydranths promise results of interest.

General conclusions.—The fact may be emphasized that the experimental data constitute undeniable evidence for the existence of a quantitative physiological differential of some sort in the *Corymorpha* stem. This evidence consists both in the different frequencies of hydranth development from incisions at different levels and

in the differences in effectiveness of grafts taken from different levels in determining development of new axes. Also, the reality and effectiveness of physiological dominance is very clearly demonstrated, both in the development of new axes from the grafts and the host tissue and from regions of incision and in the action of the distal hydranth in preventing development of new axes from grafts and incisions. The experimental data indicate that the origin of a dominant region is not dependent upon a specific organization. The organization which develops in the dominant region is the result, not the cause, of dominance. Dominance apparently depends on degree, or intensity, and duration of physiological activity in the cell mass concerned. If this is above a certain threshold probably both absolute and relative, the dominant region determines development of a hydranth and stem. At lower levels of activity it may determine development of a basal end or merely of a stem region.

SUMMARY

1. The present paper, a continuation of earlier experiments on grafting in *Corymorpha*, is primarily a study of the frequency of determination of development of new axes by lateral grafts and incisions in relation to level of origin of graft, level of implantation or incision, and dominance of the distal hydranth.

2. Very small pieces, $\frac{1}{4}$ sectors of stem pieces 1–2 mm. long, were used as grafts in all experiments for comparison of different levels. In the hydranth determined by such a graft, the graft forms only the apical region, other parts developing from the host tissue.

3. Grafts were made in series of twenty or thirty with the host hydranth present and with host hydranth removed at the time of grafting, for each of the following combinations of level of the naked stem region of donor and host: distal on distal, proximal on distal, distal on middle, proximal on middle, distal on proximal, and proximal on proximal. Lateral incisions without grafts were also made as controls in series of twenty or thirty with, and without, the distal hydranths at the distal, middle, and proximal levels.

4. The dominance of the distal hydranth is an important factor in determining the frequency of development of a new axis from either a graft or an incision. In the presence of the hydranth the fre-

quency of hydranth development from grafts is 33 to 75 per cent less than when the distal hydranth is removed at the time of grafting. No case of hydranth development from an incision without graft has been observed when the distal hydranth was present; but when the hydranth was removed at the time of incision, the frequency of hydranth development was 56.7 per cent at the distal, 26.7 per cent at the middle, and zero at the proximal level.

5. In the absence of the host hydranth, grafts from the distal donor level are more effective in determining development of a new axis than grafts from the proximal donor level.

6. As regards level of implantation, the results are complicated by the development of new axes at the distal and middle levels from incisions as well as from grafts, and by the poor conditions of material available for grafts at the proximal host level. Nevertheless, the grafts from the distal donor level show the least increase in frequency of new axes over the controls at the distal, a greater increase at the middle, and the greatest at the proximal level. Grafts from the proximal donor level show a similar increase in frequency of new axes from distal to middle level, but no further increase at the proximal level.

7. The grafts of the $\frac{1}{4}$ sectors of the stem pieces, which were made without orientation, and supplementary experiments, consisting of grafts of two $\frac{1}{2}$ sectors of stem pieces and grafts of aggregates of dissociated cells of the stem, all indicate that the original polarity of the graft is not necessarily a factor in the origin of the new polarity. In grafts of sectors of reconstituting hydranths and of transverse pieces of manubrium the original polarity may persist to a greater or less degree in the new axis.

8. All the experimental data favor the view that the determining, inducing, organizing action or dominance of the graft is dependent rather on its physiological activity than on any specific organization or specialization of the cells.

9. The differences in effectiveness in determining new axes of incisions at different levels of the stem and of the grafts in relation to level of origin and level of implantation indicate very clearly the existence in the stem of a quantitative differential or gradient in physiological condition.

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